Sealing and Healing of Fetal Membranes

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Zusammenfassung

Der vorzeitige Blasensprung vor der 37 Schwangerschaftswoche, tritt in ungefähr 1 % aller Schwangerschaften auf und ist die Ursache von 30-40 % aller Frühgeburten. Der vorzeitige Blasensprung ist somit eine der wichtigsten Ursachen für eine Frühgeburt. Sowohl der vorzeitige Blasensprung als auch die Frühgeburt erhöhen das Risiko der mütterlichen Morbidität sowie der neugeborenen Morbidität und Mortalität. Verschiedene Therapien für einen vorzeitigen Blasensprung sind vorgeschlagen und in menschlichen, fötalen Membranmodellen getestet worden. Bisher hat aber keine dieser Therapieversuche den Sprung in die klinische Praxis geschafft.

Das Ziel dieser Arbeit war es, eine alternative Therapieoption für den vorzeitigen Blasensprung mittels einer durch Tissue-Engineering hergestellten Matrix zu entwickeln. Diese Matrix sollte die natürliche Struktur des Amnions imitieren.

Zu Beginn der Arbeit wurde eine reproduzierbare Methodik etabliert, welche die Extraktion und Expansion von humanen Amnionepithel- und Amnionmesenchymzellen von Früh- und Termingeburten zulässt. In einem *in vitro* Wundheilungstest, wurde gezeigt, dass das Reparaturpotential von humanen Amnionepithel- und Amnionmesenchymzellen sowie die Reaktion auf verschiedene, Zellteilung stimulierende Faktoren, vom Gestationsalter abhängig ist. Besonders Amnionmesenchymzellen von Frühgeburten zeigen im Vergleich zu Amnionmesenchymalzellen von Termingeburten ein zweifach gesteigertes Reparaturpotential. Die Proliferation von Amnionmesenchymzellen, die aus Frühgeburten extrahiert wurden, konnte mittels Platelet Derived Growth Factor und Tumor Necrosis Factor α verdoppelt werden. Zum Abschluss der Arbeit wurden 3D

Fibrinmatrices als Gerüste für isolierte Amnionepithel- und Amnionmesenchymzellen von Frühgeburten verwendet. Amnionmesenchymzellen wurden in die Fibrinmatrix eingebettet und Amnionepithelzellen wurden auf diese Matrix plaziert. Diese Architektur ähnelt sehr der natürlichen Struktur des Amnions. Durch Hinzufügung von spezifischem extrazellulären Matrixmolekülen (Kollagen I, Laminin-1 oder Fibronektin), welche die Zelladhäsion vermitteln konnten die biologischen Eigenschaften der Fibrinmatrices verändert werden. Ausserdem stimulierten verschiedene extrazelluäre Matrixmoleküle den Matrixabbau durch zellgebundene Proteasen: Matrix Metalloproteasen oder Serinproteasen (zum Beispiel Plasmin). Es wurde gezeigt, dass Amnionepithel- und Amnionmesenchymzellen von Frühgeburten in solchen modifizierten 3D Fibrinmatrices ihre natürliche Morphologie und Lebensfähigkeit behalten. Ausserdem wurde Kollagen I als Grundgerüst für eine 3D Matrix untersucht. Ein wesentliches Problem in diesem Modell war die Schrumpfung und folglich die Grössenreduktion der Kollagen I Matrix *in vitro*. *In vivo* würde eine solche Schrumpfung eines Implantates zu einem unvollständigen Amnionverschluss mit den entsprechenden klinischen Konsequenzen führen. Im Gegensatz zur Kollagen I Zellmatrix ergab eine Fibrin basierte Zellmatrix *in vitro* während einer Woche keine Schrumpfung.

Die Daten dieser Arbeit, lassen vermuten, dass fibrinbasierte Zellmatrix-Systeme bei der Transplantation von Amnionzellen nützlich sein könnten. Jedoch müssen diese Daten als vorläufig betrachtet werden. Die nächsten Untersuchungen zielen nun daraufhin, dies mittels Tissue-Engeneering hergestellten Amnionzelltransplatate unter komplizierteren, multifaktoriellen Bedingungen zu testen.

Summary

Premature rupture of the membranes occurring before 37 weeks' gestation is usually referred to as preterm premature rupture of the membranes (PPROM). PPROM occurs in approximately 1 % of all pregnancies and is associated with 30-40 % of preterm deliveries. It is the leading single identifiable cause of preterm delivery known so far. Both PPROM and preterm birth carry a high risk of maternal morbidity and neonatal morbidity and mortality. Several treatments for PPROM in human fetal membrane models have been proposed but none of these treatments have yet been introduced into clinical routine.

This thesis focuses on a strategy to develop a potential option for the treatment of PPROM patients based on a tissue engineering approach. In particular, this approach relies on the design of a cell containing matrix that mimics the architecture of native amnion.

First, a reproducible methodology that permits to collect and expand human amnion epithelial and mesenchymal cells from preterm and term placenta was established. Furthermore, using an *in vitro* lesion repair assay, it was demonstrated that the repair potential of human amnion epithelial and mesenchymal cells as well as their reaction towards different proliferation stimulants depends on the gestational age. Especially, preterm amnion mesenchymal cells showed approximately two fold increased repair potential compared to their term counterparts. Moreover, an increase in the proliferation of preterm amnion mesenchymal cells was found after treatment with platelet derived growth factor and tumor necrosis factor α. Furthermore, 3D fibrin matrices were used as a scaffold for isolated preterm amnion epithelial and mesenchymal cells.

As outlined in this thesis, an interesting refinement of the here introduced 3D fibrin-matrix system could be the addition of specific peptide sequences involved in cell adhesion and/or migration, and being degradable by cell-associated proteases such as matrix metalloproteinases or serine proteases such as plasmin. It was demonstrated that preterm amnion epithelial and mesenchymal cells in modified 3D fibrin based cell-matrix systems acquired their natural morphologies and displayed viability. Furthermore, the use of collagen I as a scaffold matrix was also investigated in this work. Limitations of this approach include contraction and consequently size reduction of collagen I matrices *in vitro*. *In vivo* such size reduction would inevitably induce failure of the implant followed by severe clinical consequences. In contrast to collagen I matrix-cell systems, no decrease in size of fibrin cell-matrix systems was observed over the time course of one week, suggesting also size stability for *in vivo* use.

Related to this data, one might speculate, that such fibrin based cell-matrix systems may be useful in amnion cell transplantation. However, these data are clearly preliminary and have to be confirmed by exposure of amnion cell-based tissue engineered grafts to more complex multifactorial *in vivo* conditions.

Table of contents

Chapter 1

General introduction

Background

All verterbrates have accessory extra-embryonic tissues enclosing the fetus, known collectively as the fetal membranes. These tissues comprise the three primary germ layers that are not innervated. These layers only exist as embryonic accessories. They are genetically identical to the fetus, but have a limited life time, existing only until the fetus is developed sufficiently for it to become a functional individual. The embryonic development of the fetal membranes begins as soon as the blastocyst implants into the maternal endometrium. Most of the blastocyst's cells constitute the outer wall (trophoblast), surrounding the blastocyst cavity. Generally speaking, the trophoblast is the preceding tissue to the fetal membranes, including the placenta. The inner cell mass, a small group of larger cells that form the embryoblast, is apposed to the inner surface of the trophoblastic vesicle. The embryo, the umbilical cord, and amnion are derived from these cells. During attachment and after invasion of the endometrial epithelium, the trophoblastic cells of the implanting embryonic pole of the blastocyst show increased proliferation, resulting in a double-layered trophoblast. The outer of the two layers directly facing the maternal tissue, is transformed to a syncytiotrophoblast by fusion of neighbouring trophoblast cells. The remaining cellular components of the blastocyst wall, which have not yet achieved contact to maternal tissues are called cytotrophoblast. Between day 13 and 14 post coitus, the cytotrophoblast proliferates to form cellular columns invading the syncytiotrophoblast (primary villi). Further proliferation and branching results in the formation of the secondary and tertiary villi (Figure 1). The network of branching villi suspends the chorion cavity of the endometrium. Further expansion obliterates the capillaries and leads to degeneration of the villi in the chorion and the chorion becomes smooth (chorion leave). Simultaneously, there is proliferation of villi at the decidua basalis, where the fetal part of the placenta arises (chorion frondosum). The 'chorion leave' is usually considered as synonymous for 'membranes' and is distinct from the 'chorion frondosum', which is actually placental tissue.

Fetal membranes are highly specialized areas of maternal-fetal interactions, which appear to have achieved greater significance in the higher vertebrates for the survival of the pregnancy, as well as for parturition[1]. The extraplacental fetal membranes, amnion and chorion, have also been described as individual membranes[2, 3] (Figure 2).

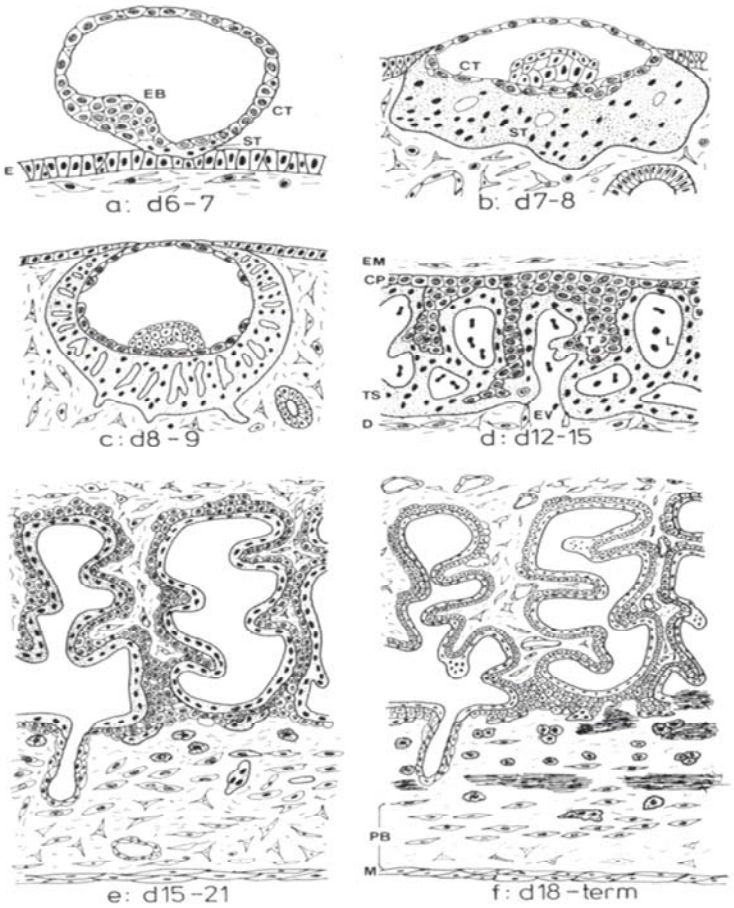


Figure 1. Simplified drawings of typical stages of early placental development. (a&b) Prelacunar stages. (c) Lacunar stage. (d) Transition from lacunar to primary villous stage. E=endometrial epithelium; EB=embryoblast; CT=cytotrophoblast; ST=syncytiotrophoblast; EM=extraembrionic mesoderm; CP=primary chorionic plate; T=trabeculae and primary villi; L=maternal blood lacunae; TS=trophoblastic shell; EV=endometrial vessel; D=deciduas; PB=placental bed. Adopted from Benirschke and Kaufmann (1990)[1].

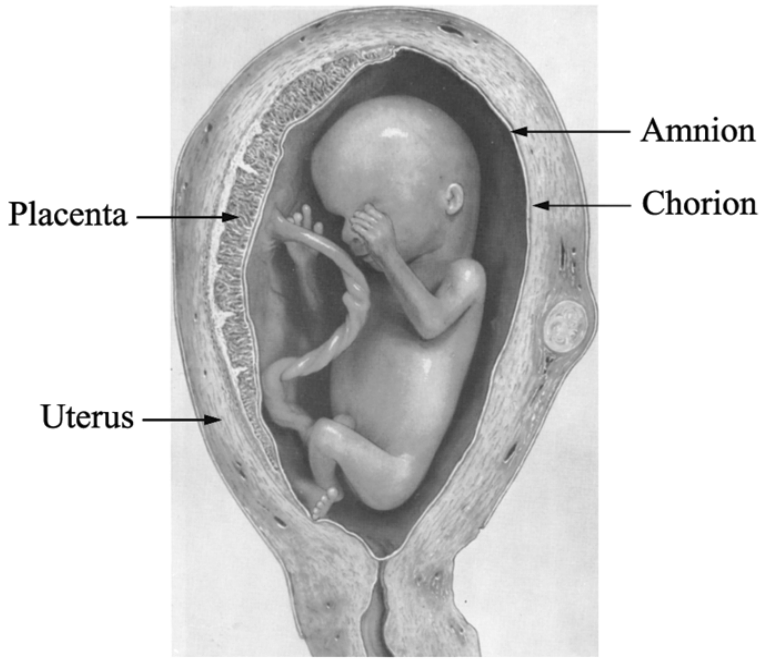
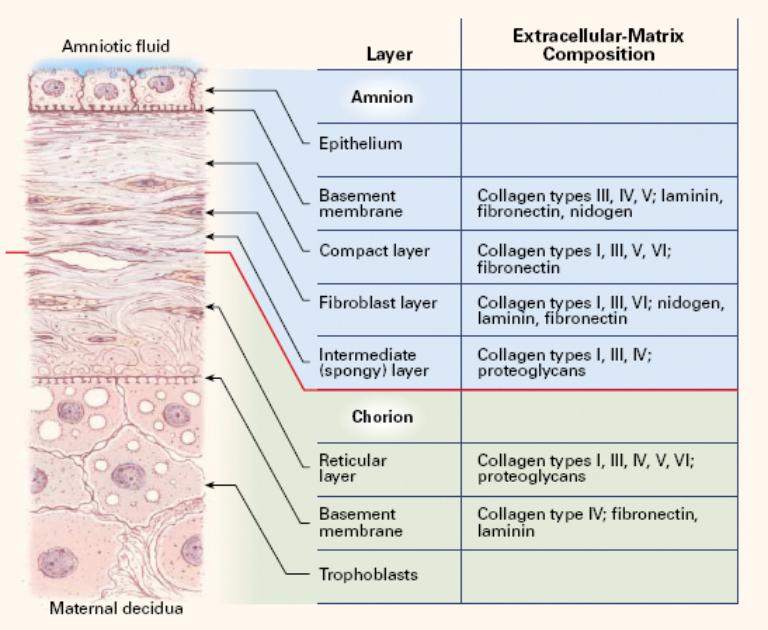


Figure 2. Photograph of a sagittal section of a uterus containing a fetus. Adapted from Boyd (1970)[4].

Structure of human fetal membranes

Measuring only 0.08 to 0.12 mm in thickness, the human amnion is composed of five morphologically distinct layers (Figure 3). It contains no blood vessels or nerves; the nutrients it requires are supplied directly by diffusion out of amniotic fluid and/or from the underlining decidua. The innermost layer, nearest to the fetus, is named amniotic epithelium. Amniotic epithelial cells secrete collagen types III and IV and noncollagenous glycoproteins (laminins, nidogen, and fibronectin) that form a basement membrane. This basement membrane constitutes the second layer of the amnion. The basement membrane provides a solid support for amniotic epithelial cells. The compact layer of connective tissue adjacent to the basement membrane forms the main fibrous skeleton of the amnion. The collagens of the compact layer are secreted by

mesenchymal cells situated in the fibroblast layer. Interstitial collagens (types I and III) predominate and form parallel bundles that maintain the mechanical integrity of the amnion. Collagen types V and VI form filamentous connections between interstitial collagens and the epithelial basement membrane. The fibroblast layer is the thickest of all amniotic layers. The intermediate layer (spongy layer, or zona spongiosa) lies between amnion and chorion. Its abundant content of proteoglycans and glycoproteins produces a 'spongy' appearance in histologic preparations, and it contains a nonfibrillar meshwork of mostly type III collagen. The intermediate layer adsorbs physical stresses by permitting the amnion to slide relative to the underlying chorion, which is firmly adherent to the maternal decidua. Although the chorion (0.04 to 0.4 mm in thickness) is approximately 4 times thicker than the amnion, the amnion has greater tensile strength. The chorion resembles a typical epithelial membrane, with its polarity directed towards the maternal decidua. As pregnancy progresses, trophoblastic villi within the chorionic layer of the fetal membranes that are free of the placenta, regress[5].



Layer	Extracellular-Matrix Composition
Amnion	
Epithelium	
Basement membrane	Collagen types III, IV, V; laminin, fibronectin, nidogen
Compact layer	Collagen types I, III, V, VI; fibronectin
Fibroblast layer	Collagen types I, III, VI; nidogen, laminin, fibronectin
Intermediate (spongy) layer	Collagen types I, III, IV; proteoglycans
Chorion	
Reticular layer	Collagen types I, III, IV, V, VI; proteoglycans
Basement membrane	Collagen type IV; fibronectin, laminin
Trophoblasts	

Figure 3. Schematic representation of the structure of the fetal membranes at term. The extracellular-matrix composition of each layer is shown. Adopted from Parry et al. (1998)[5].

Human amnion epithelial and mesenchymal cells

Early in embryogenesis, i.e. before 8 weeks of gestation, the amnion membrane is comprised of a layer of epithelial cells (presumably derived from the fetal ectoderm) and a separate layer of mesenchymal cells (presumably derived from the fetal mesoderm) that lie immediately adjacent to the epithelial cells. At this early stage of embryogenesis, there are approximately equal numbers of amnion epithelial and mesenchymal cells. As the amniontic sac expands, the epithelial cells replicate at a rate sufficient to maintain a continuous layer of epithelium. The cells are connected by desmosomes. The rate of replication of the mesenchymal cells, however, apparently does not keep pace with the expansion of the amniontic sac. By 10-14 weeks gestation, these fibroblast-like cells begin to be dispersed, ultimately being connected only by a loose lattice of connective tissue. During the third trimester of pregnancy, there are, on average, only about one-tenth as many mesenchymal as epithelial cells[6].

Human amnion epithelial cells are immunologically naïve because they do not express any of the leukocyte antigens (HLA)-A, -B, -C or –DR[7]. Immune reaction did not occur after transplantation in volunteers[8]. Since then, amniotic tissue has been used for allotransplantation to treat patients with chronic sphingomyelinase deficiency[9] and lysosomal storage disease[10]. In addition, it has already been reported that human amnion epithelial cells may have a significant role in supplying neurotrophic factors as well as neurotransmitters to the amniotic fluid, suggesting an important function in the early stages of neural development of the embryo. Human amnion epithelial cells present immunological markers for neurons, astrocytes and oligodendrocytes, as they synthesize and release acetylcholine and catecholamine as well as express mRNA encoding for dopamine receptors and transporters[10].

Almost all studies describing amnion cellular function have been conducted with amnion epithelial cells, however, amnion mesenchymal cells have largely been ignored. There is no clarity concerning the immunogenicity of amnion mesenchymal cells, since transplantation studies have never been performed. Recently, it has been shown that some critical functions of the amnion are performed by mesenchymal cells. For example, interstitial collagens that provide the strength of the amnion are synthesized and processed exclusively in mesenchymal cells. The enzyme lysyl oxidase, which

catalyses the initial reaction in the cross-linking of interstitial collagen fibrils, is also expressed primarily in these cells. Furthermore, the mesenchymal cells are the major source of tissue inhibitor of metalloproteinase-1 (TIMP-1), cytokines and produces keratinocyte growth factor (KGF) which is involved in wound repair[11].

Functions of human fetal membranes

Human pregnancy and parturition present a unique set of challenges for fetal membranes, which form an adjustable biomechanical container for a large growing and moving of fetus[12]. Sufficient strength and elasticity are needed to withstand slow but progressive stretching to approximately double their size by term and simultaneously to protect against rapid pressure on the maternal abdomen. The spongy layer is rich in proteoglycans which contains >90 % water and swell, allowing the amnion to slide relative to the chorion. Amnion and chorion retain amniotic fluid, secrete substances both into the amniotic fluid and towards the uterus. The fetal membranes allow passive diffusion of electrically neutral, lipophilic substances, oxygen, electrolytes and water. Certain constituents, such as amino acids, iron, calcium, and phosphorus, enter by active transport. Glucose penetrates by facilitated diffusion[13]. Furthermore, the membranes protect the fetus against infections ascending the reproductive tract. During the pregnancy the major functions of amnion and chorion are maintenance and protection of the fetus, however at the end of the pregnancy, the fetal membranes must undergo planned degradation allowing the delivery of the fetus at term. The membranes need to be receptive to endocrine and paracrine signals from mother, fetus and the materno-fetal interface.

Rupture of human fetal membranes

Fetal membranes normally rupture during labour (uterine contraction). For most pregnancies, labour starts at 38-42 weeks gestation in the presence of intact membranes. The events leading to fetal membrane weakening and rupture at term are not fully understood. A cascade of events involving mechanical membrane distortion, extracellular matrix distortion with loss of cell-matrix interactions, apoptosis, MMP-activation, and membrane degradation has been proposed and is supported by ample experimental material[5, 14]. Rupture at term before the onset of regular labour is defined as premature rupture of membranes (PROM). It takes place in some 8-10 % of pregnancies. Premature rupture of the membranes occurring before 37 weeks' gestation is usually referred to as preterm premature rupture of the membranes (PPROM). PPROM occurs in approximately 1 % of all pregnancies and is associated with 30-40 % of preterm deliveries. It is thus the leading single identifiable cause of preterm delivery[5]. High fetal morbidity and mortality rates occur with PPROM because of infection, premature labor, fetal compromise from umbilical cord compression, and/or fetal deformation (pulmonary hypoplasia and/or arthrogryposis). Maternal complications are also more common with PPROM, including chorioamnionitis rates as high as 25-35 %[15].

The term iatrogenic PPROM (iPPOM) was induced to describe PPROM as a result of an invasive intrauterine procedure like amniocentesis or fetoscopy, during which, by definition, the amnion cavity is entered and therefore the membranes disrupted[16]. iPPROM is a major limitation of uterine endoscopy. The incidence of iPPROM is 10 % after laser coagulation for twin-to-twin transfusion syndrome[17], >30 % after fetoscopic cord ligation[18] and >62 % after endoscopic tracheal clipping[19]. Despite advances in perinatal care, PROM and PPROM continue to be important obstetrical complications.

The cause of premature rupture of the fetal membranes is almost certainly multifactorial (Figure 4). Traditionally, rupture of the fetal membranes has been attributed to increasing physical stresses that weaken the membranes. At the molecular level, premature rupture of the membranes appears to result from diminished collagen synthesis, altered collagen structure, and accelerated collagen degradation, possibly in

association with concurrent cellular changes within the fetal membranes. These hypotheses are not mutually exclusive, and biophysical stresses may amplify these biochemical changes[5].

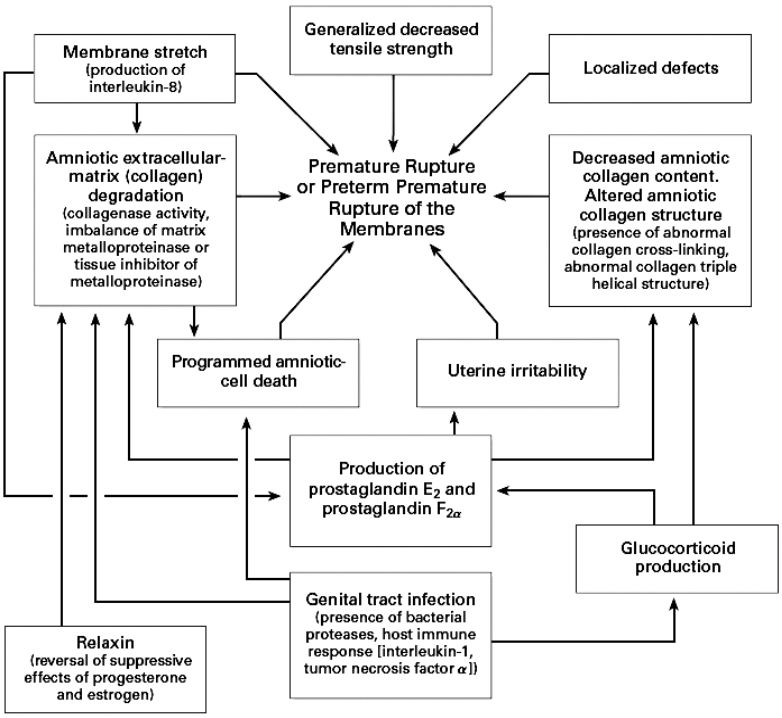


Figure 4. Schematic diagram of various mechanisms that have been proposed to result in PROM or PPROM. Taken from Parry et al. (1998)[5].

Fetal membranes: healing and sealing

Spontaneous wound healing in fetal membranes

Knowledge about the healing potential of fetal membranes has long been limited to case reports related to iatrogenic rupture after amniocentesis. Older case-reports mention the presence of defects in the fetal membrane several weeks after invasive procedures[20, 21]. However, most cases of post-amniocentesis amniorrhexis are self-limited, resealing spontaneously with favourable pregnancy outcomes[22, 23]. Resealing of the membranes

following spontaneous PPROM (sPPROM) is less frequent and occurs in only 7.7 to 9.7 % of cases[24, 25]. This suggests that the fetal membranes have the capacity under certain, very individual conditions to heal a created or spontaneously occurring defect. However, the defect might be concealed or the membrane could re-seal through retraction, sliding, contraction and scarring in the myometrial and decidual layers of the uterus, rather than involving an active healing mechanism at the level of the fetal membranes[26].

Surgical sealing of fetal membranes: Clinical approaches

Actual expectant management of PROM differs from institution to institution and includes immediate delivery or termination of pregnancy versus conservative management with or without the use of tocolytics and steroids in various combinations[27].

Several methods for sealing and healing of human fetal membrane defects have been proposed, but clinical series are small and their successes limited. So far, none of these techniques have been introduced into routine clinical practice[28].

One of the first case reports refers to the successful application of maternal blood to the amnion leakage under ultrasonographic guidance to produce a clot patch. Leakage stopped within 12 h and ultrasonographic investigation showed that the blood clot was gradually diminished and completely disappeared within 3 weeks[29]. A healthy baby was born at term. This was an individual case and the application was performed in a patient with iPPROM following amniocentesis at 16 weeks gestation.

Some cases of apparent successful sealing of fetal membranes were reported after iPPROM with a proportion of platelets injected transabdominally into the amniotic cavity along with a proportion of cryoprecipitates (so-called amniopatch)[30, 31]. This was the first successful treatment of iPPROM in a case with persistent amniotic fluid leakage following fetoscopy at 18 weeks of gestation. The hypothesis was that platelets became activated at the rupture site and fibrin clot formation would initiate the healing process. Such a procedure would first allow the membranes to seal and then to be restored by surrounding amnion cells. However the detailed mechanism by which this

amnion patch was successful is not understood, since the amnion patch was injected into the amniotic cavity without being aware of the exact localisation of the rupture. It is not clear how the material could find its own way to the defect and seal it.

In this context, the interaction between platelets and the fetal membranes has been studied in vitro[32]. It was found that exposed connective tissue of amniotic membranes triggered platelet adhesion, aggregation, as well as activation. Platelets were shown to seal a standardized puncture of fetal membranes under a certain pressure. However, it is still not clear, whether this platelet-fibrin plug effectively stimulates tissue regeneration at the trauma site. Furthermore, the original in vivo reports, mentioned above, have some important limitations. Two of the six cases were complicated by sudden intrauterine fetal dead. Severe bradycardia and hypotension have been observed following platelet transfusion. Thus, it has been hypothesized that hemodynamic changes secondary to platelet activation could have caused the fetal demise. Given these serious complications, application of amnion patches to seal leakage of fetal membranes is not satisfying to allow clinical application. Further experimental work is needed to determine the optimal origin, composition, volume and concentration of blood products administered.

Several authors developed strategies to attempt sealing of membranes undergoing sPPROM[33-37] using fibrin sealants. Fibrin sealants were applied in order to act as a cervical plug to stop further amniorrhexis and prevent ascending infections. Inconsistent results, small number of cases and poor study design without control groups makes it difficult to draw meaningful conclusions. The continuation of that work was a case report describing the direct application of a high concentration of maternal platelets and fibrin glue to the uterine surface of the damaged amniotic membrane under fetoscopic visualization. The envisioned mechanism of the procedure was formation of a platelet-fibrin plug which acutely blocks further leakage of fluid and may offer a lattice for amniocyte migration and final closure[38]. Doubts have to be expressed on these studies since amniorrhexis reappeared weeks after the procedure[30].

In vitro, fibrin glue effectively improved the structural integrity of artificially punctured human chorioamniotic membranes. Different fibrin sealant compositions were compared for their efficacy to seal a standardized puncture in fetal membranes.

Amniotic membranes were best sealed by a fibrin/thrombin-based sealant[39]. Both, fibrinogen and thrombin are required to form a fibrin clot in the final step of the clotting cascade. Some in vivo results exist about the transvaginal application of intracervical fibrin sealants[40]. Fibrin tissue sealants were shown to increase the postrupture latency period and neonatal survival. The fibrin clot was probably able to seal and interrupt the leakage of amniotic fluid, but this was not in all cases accompanied with an increase in the amniotic fluid volume. Fibrin sealants may further act as barriers to ascending infections, but only in cases where a preliminary placement of a cervical cerclage is omitted. Although fibrin sealants promote local growth and tissue repair it is doubtable that a direct closure of the membranes has been obtained.

In a rabbit model fetoscopic access sites were successfully sealed with collagen plugs[41]. The use of collagen plugs in combination with suturing of the myometrial layer resulted in functional restoration of the membrane integrity with preservation of amniotic fluid and normal fetal pulmonary growth in more than 80 % of the cases. Histological examination revealed entrapment of membranes between the plug and the myometrium but no anatomic repair of the membranes.

Gelatine sponges were also used to close fetal membrane defects in vitro[42]. The primary benefit of this material turned out to be the length of time it remained intact within the amnion cavity. Gelatine sponges remained longer at the wound site than fibrin or other blood products. A similar method was used in sheeps and primates, when gelatine sponges were used to maintain membrane integrity after fetoscopy[43]. The technique was furthermore evaluated in vivo, in patients with the poorest outcomes based on prognostic factors. After cervical cerclage, a gelatine sponge was administered into the amniotic cavity. Within only 30 % surviving infants, the in vitro expectations were not confirmed. Major concerns were potential fetal aspiration of the implanted gelatine sponge or obstruction of the fetal gastrointestinal tract[44, 45].

The term amnion graft was established by a case report[46]. It describes a commercial collagen plug, placed endoscopically over the membrane defect in a case of spontaneous PPROM, followed by fixation with fibrin adhesive. Gluing with fibrin proved quite effective but required expansion of the amniotic cavity with carbon dioxide, which can further dissect the chorionic space. Endoscopically, the clinical characteristics of the site

of rupture suggested that the size, location and shape of the defect were unlikely to be healed with an amniopatch.

Concluding, it has to be pointed out again that all of the mentioned techniques are far from being introduced into the routine clinics. Therefore, alternative methods have to be explored to manage either sPPROM or iPPROM and to decrease the likelihood of miscarriage, preterm delivery and the complication of prematurity.

Tissue Engineering - Regenerative Medicine

Tissue engineering is an interdisciplinary field in which the principles and methods of engineering combine with those of biological sciences for the fundamental understanding of structure-function relationships in normal and pathological tissues and organs, as well as for the development of biologic substitutes that can restore, maintain, or improve tissue or organ function. More recently, the expression 'regenerative medicine' has been often used either as a synonym or as an all-embracing branch of medical science that would include tissue engineering per se.

In the current context of tissue engineering, as defined above, new tissues or organs can be created through three general strategies, or a combination of them, as follows:

(1) Infusion of isolated cells, i.e. cells are isolated from donors, expanded and/or modified in vitro and re-implanted for the supply of a specific function.

(2) Tissue-inducing substances. In this process, appropriate signal molecules (e.g. growth factors) are delivered to specific targets for the stimulation or control of tissue growth or maturation.

(3) Cells placed on or within performed scaffold (matrices). This methodology consists of culturing the cells on or within a natural or synthetic but degradable scaffold matrix *in vitro* and transplantation of the cell-matrix composite at the site where regeneration is required. The scaffold material provides initial mechanical support and a template for three-dimensional organization.

All of these strategies can be applied in autologous, heterologous, or xenologous manner. They all carry potential problems, enclosing immunologic rejection, growth

limitations, differentiation and function restrictions, incorporation barriers and cell or tissue delivery difficulties. Despite the limited clinical experience to date, efforts at engineering almost every mammalian tissue have already occured[47].

Acute wound healing: an example for tissue regeneration

Healing of an acute wound follows a predictable chain of events. This chain of events occurs in a carefully regulated manner that is reproducible from wound to wound. The phases of wound healing are overlapping, but are described in a linear fashion for the purpose of clarity. The five phases that characterize wound healing include (1) hemostasis, (2) inflammation, (3) cellular migration and proliferation, (4) protein synthesis and wound contraction, and (5) remodelling (Figure 5)[48].

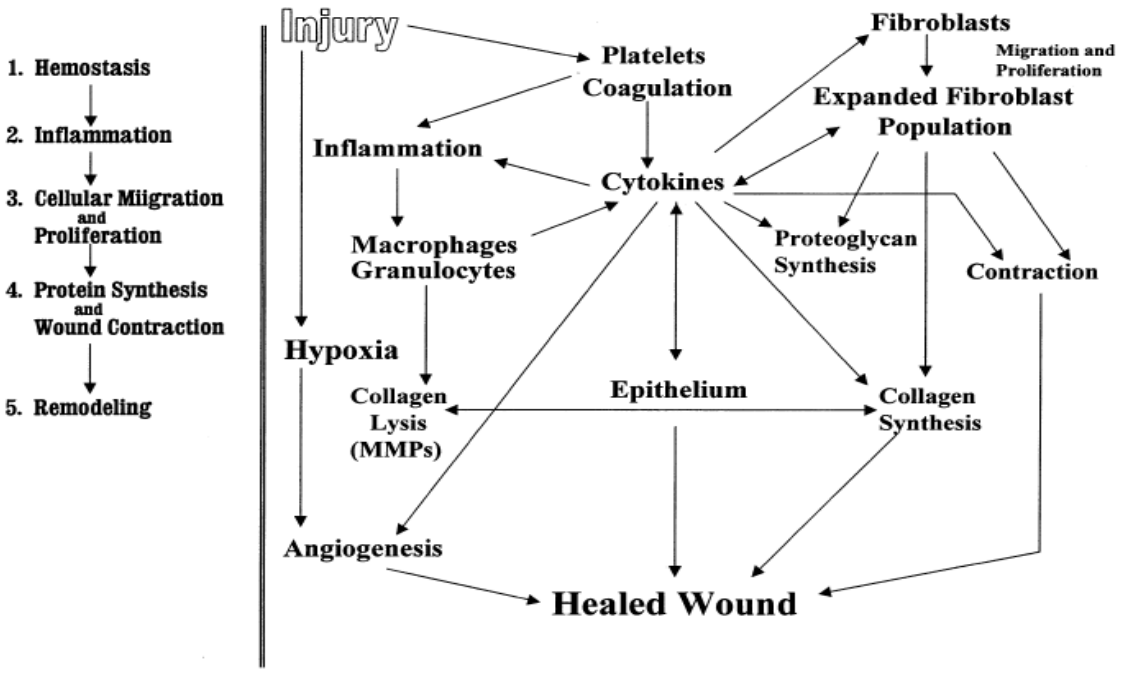


Figure 5. The wound-healing cascade of an acute trauma. The progression of acute wound healing from hemostasis to the final phases of matrix remodelling is dependent on a complex interplay of varied acute wound-healing events. Cytokines play a central role in wound healing and serve as a central signal for various cell types and healing events. Taken from Monaco et al. (2003)[48].

Hemostasis

All significant trauma create a vascular injury and thereby initiate the molecular and cellular responses that establish hemostasis. The healing process cannot proceed until hemostasis is accomplished. Primary contributors to hemostasis include vasoconstriction, platelet aggregation, and fibrin deposition resulting from the coagulation cascades. Vasoconstriction is initiated by the release of vasoactive amines, which occurs when the dermis is penetrated. Epinephrine is released into the peripheral circulation, whereas stimulation of the sympathetic nervous system results in local norepinephrine release. Injured cells secrete prostaglandins, such as thromboxane, that contribute further to vasoconstriction. In wound healing, platelets are the first cells to arrive at the site of injury. Mediated by the integrin $\alpha_{IIb}\beta_3$, adult platelets adhere to wound connective tissue. They are activated by contact with exposed collagen and collagen fragments to discharge their alpha granules and aggregate to form a platelet plug. Adhesion proteins (such as fibrinogen, fibronectin, thrombospondin, and von Willebrand factor) and growth factors (such as platelet-derived growth factor [PDGF] and transforming growth factor-β [TGF-β]) are released from the alpha granules and promote further platelet adhesion and aggregation. The end product of the hemostatic process is clot formation. Clots are primarily composed of a fibrin mesh containing aggregated platelets and blood cells. The coagulation cascades are composed of intrinsic and extrinsic components that are individually triggered (Figure 6)[49]. The intrinsic coagulation cascade is initiated by activation of factor XII (FXII), which occurs when blood is exposed to foreign surfaces. The release of tissue thromboplastin factor (TF) from damaged tissue initiates the extrinsic coagulation pathway. Tissue thromboplastin factor binds and activates coagulation factor VII (FVII). The TF-FVIIa complex subsequently acts as a potent activator of coagulation factor IX (FIX) and factor X (FX) in the presence of calcium (Ca^{2+}), resulting in the formation of FIXa and FXa.

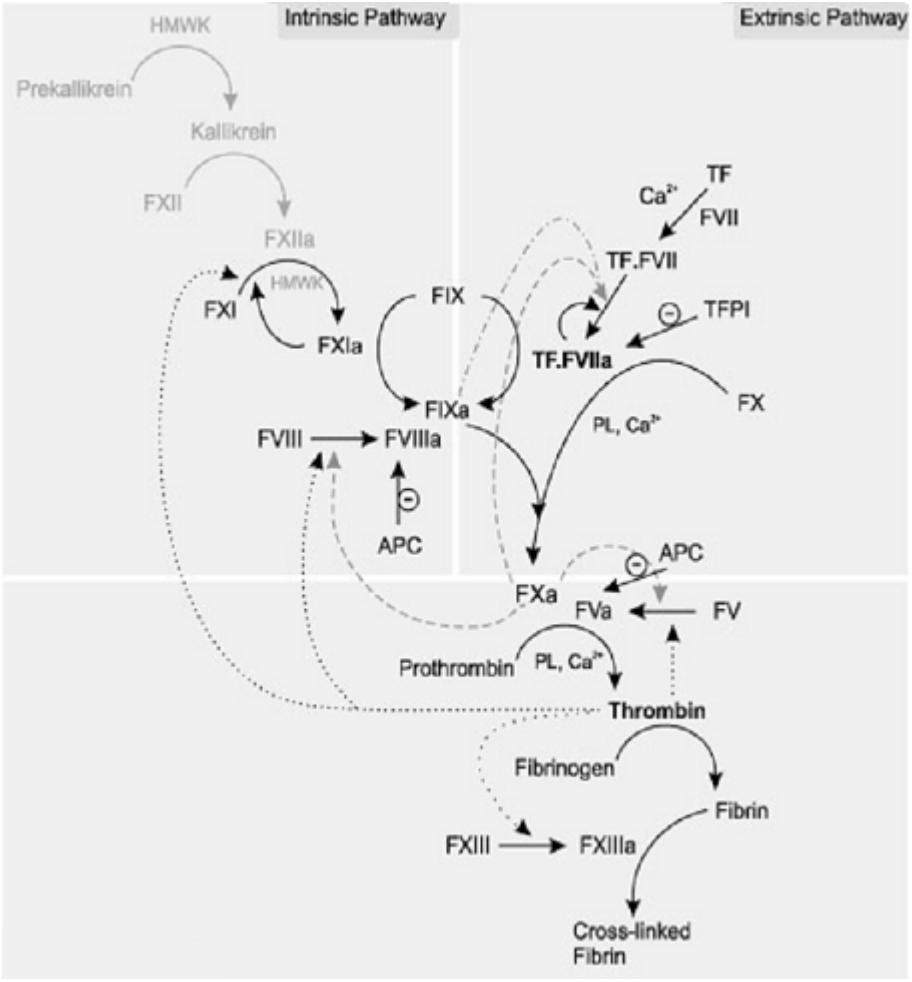


Figure 6. Schematic overview of the blood coagulation cascade. The model is divided into the intrinsic (left side) and extrinsic (right side) pathway. The blood coagulation factors are denoted in Roman numbers. Active forms are denoted by a small 'a' added to the Roman number. TF, tissue factor; PL, phospholipid; HMWK, high molecular weight kininogen. Positive feedback loops by thrombin (dotted lines), FIXa (dashed-dotted line), and FXa (dashed line) are indicated in grey. Y indicates inhibition by activated protein C (APC) and tissue factor pathway inhibitor (TFPI). Adapted from Spronk et al. (2003)[50].

Activation of both the extrinsic and the intrinsic pathways of coagulation leads to formation of FIXa which, in the presence of phospholipids, calcium, and activated coagulation factor VIII (FVIIIa), is a potent activator of FX. Once formed, FXa converts prothrombin into thrombin. This reaction is dependent on the formation of the prothrombinase complex that consists of FXa, activated coagulation factor V (FVa), and phospholipids, and requires calcium as cofactor. Active thrombin converts fibrinogen into fibrin, resulting in propagated clot formation and leading to repair of the tissue injury. Another important function of thrombin is the activation of coagulation factor XIII (FXIII) to FXIIIa. This enzyme stabilizes, in the presence of calcium, the fibrin clot via covalent cross-links[49]. Fibrinogen molecules are elongated 45-nm structures consisting of two outer D domains, each connected by a coiled-coil segment to a central E domain (Figure 7). They are comprised of two sets of three polypeptide chains termed α, β, and γ which are joined together within their N terminal E domains by disulfide bridges. The thrombin-induced conversion of fibrinogen into fibrin is a complex process (simplified in Figure 7). The N-terminal region of each α chain contains a fibrinopeptide A (FPA) sequence, cleavage of which by thrombin initiates the fibrin assembly process by exposing the polymerization E site. Each E site combines with a constitutive complementary binding pocket in the D domain of neighbouring molecules.

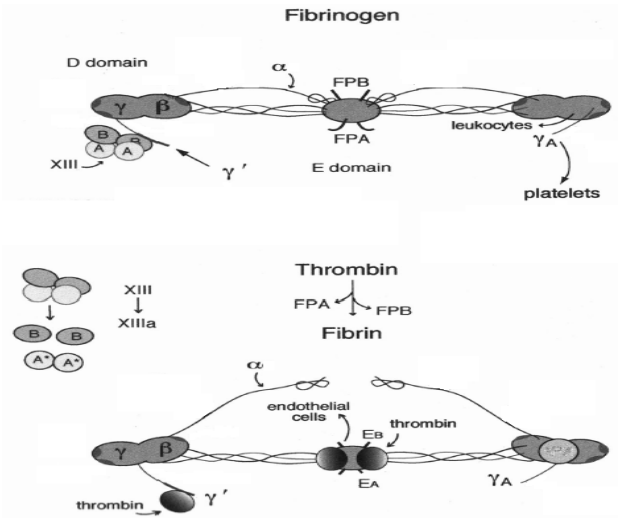


Figure 7. Schematic diagram of fibrinogen and fibrin showing the major structural domains and some cellular binding interactions. Adapted from Mosesson et al. (2001)[51].

These initial E:D associations cause fibrin molecules to align into a staggered overlapping end-to middle domain arrangement forming double-stranded twisting fibrils (Figure 8). Fibrils also undergo lateral associations to form larger diameter fibrils and the fibers constitute the three-dimensional fiber network. Two types of branch junctions occur in fibrin network structures. The first type occurs when a double-stranded fibril converges laterally with another fibril to form a four-stranded fibril, a so-called 'bilateral' junction. Lateral convergence of larger fibrils or fibers evidently result in larger versions of this type of branch junction. The second type of branch junction, termed 'equilateral', forms by the coalescence of three fibrin molecules that connect three fibrils of equal widths (Figure 8). Equilateral junctions form with greater frequency when fibrinopeptide cleavage is relatively slow. Under such conditions the networks are more branched and the matrix 'tighter' (i.e. less porous) than those formed at high levels of thrombin. Network density is dependent on the amount of thrombin, calcium, and activated coagulation factor XIII (FXIIIa)[51]. Fibrin forms the network that stabilizes the platelet plug and therefore becomes a key component of the provisional matrix that develops in the wound soon after the injury. Fibrin networks contain vitronectin derived from serum and aggregating platelets. This action facilitates the binding of fibronectins, which are produced by fibroblasts and epithelial cells. Fibronectin is the second key component of the early provisional wound matrix. The fibronectin molecule has nearly a dozen binding sites for cellular attachment. These attachment sites are essential for cellular migration along the matrix. The fibrin-fibronectin matrix also traps circulating cytokines for the use in the following stages of wound healing.

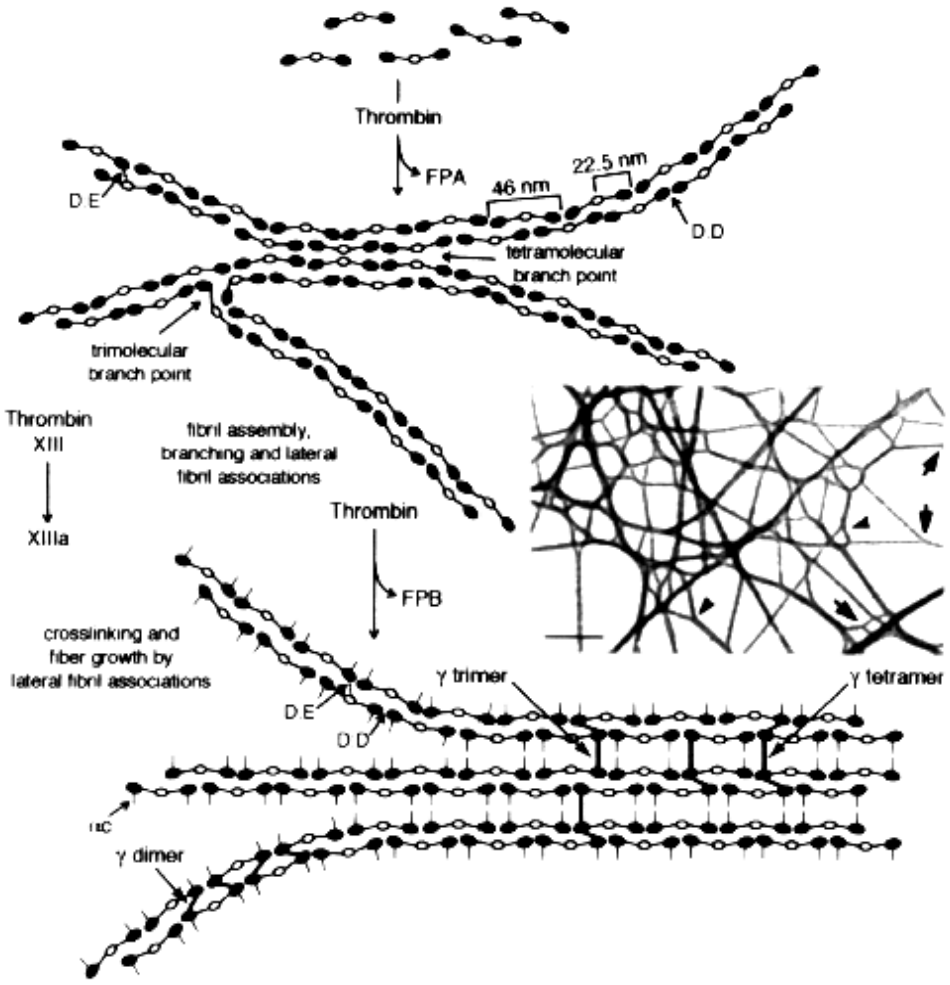


Figure 8. Schematic diagram of fibrin assembly and crosslinking. Assembly of fibrin begins with non-covalent interactions (D:E) between the E and D sites (dotted lines) to form end-to-middle staggered overlapping double-stranded fibrils (upper part of the sketch). Fibrils also branch and undergo lateral associations to form larger diameter fibrils and fibers. [Insert: critical point dried thin fibril matrix containing equilateral (arrows) and bilateral (arrowheads) branched junctions; bar, 100 nm]. Adapted from Mosesson et al. (2001)[51].

Inflammation

One of the primary functions of inflammation is to bring inflammatory cells into the injured area. These cells destroy bacteria and eliminate debris from necrotic and apoptotic cells and damaged matrix so that the repair processes can proceed. Migrating monocytes transform into macrophages as they migrate into the extravascular space in a process that is stimulated by chemotactic factors such as collagen fragments, fibronectin and elastin derived from the damaged matrix, TGF-β, complement components, enzymatically active thrombin, and serum proteins. All leukocytes require activation before they can perform their vital functions in the wound environment. Interleukin-2 (IL-2) and interferon-σ (INF-σ) derived from T lymphocytes are involved in macrophage activation. In addition to phagocytosing debris, macrophages also contribute to matrix breakdown by releasing matrix metalloproteinases (MMPs) such as collagenases and elastase into the wounded area. These multipurpose cells are involved in many aspects of healing through the cytokines and immunomodulatory factors they produce. Macrophage-produced cytokines are involved in angiogenesis, fibroblast migration and proliferation, collagen production, and possibly wound contraction. TGF-β, fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-1), PDGF, and IL-1 are several of the most crucial macrophage-derived cytokines.

Cellular migration and proliferation

The initial fibrin-fibronectin matrix is heavily populated by inflammatory cells, whereas fibroblasts and endothelial cells will predominate and accompany the healing progresses. Cytokine networks continue to be part of the following healing processes as cytokine release contributes to fibroplasia, epithelialization, and angiogenesis. Although much is known about the signals that stimulate the predominant activities during this phase of healing, less is known about the signals that bring these activities to a controlled end. Negative feedback mechanisms that deactivate cells after they have completed their tasks are also essential for normal wound healing. Moreover, fibroblasts are required in the healing wound, since native fibroblasts were lost or damaged in the injury. Repopulation of the wounded area with fibroblasts occurs as a result of fibroblast migration from adjacent tissues and proliferation of cells in the wound. In

addition, undifferentiated cells in the environment of the wound may transform into fibroblasts under the influence of cytokines in the wound milieu. Factors that stimulate fibroblast migration include TGF-β, epidermal growth factor (EGF), PDGF, and fibronectin. Upregulation of cell membrane integrin receptors that bind to fibrin and fibronectin in the provisional wound matrix is required for fibroblasts to migrate. An integrin molecule is composed of two noncovalently associated transmembrane glycoprotein subunits called α and β (Figure 9).

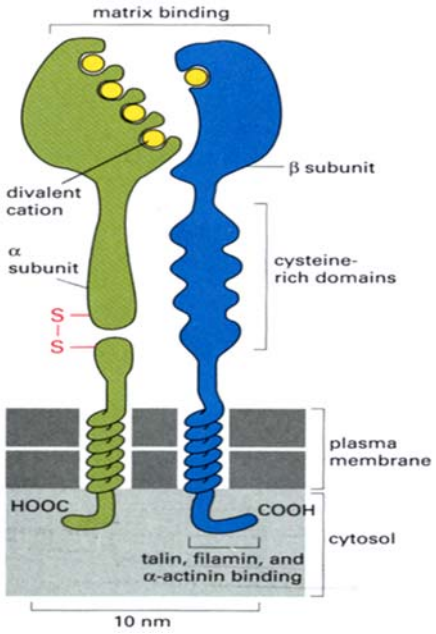


Figure 9. The subunit structure of an integrin cell-surface matrix receptor. The α and β subunits are held together by noncovalent bonds. The α subunit is made initially as a single polypeptide chain, which is then cleaved into one small transmembrane domain and one large extracellular domain that contains divalent-cation-binding sites; the two domains remain held together by a disulfide bound. The extracellular part of the β subunit contains a single divalent-cation-binding site, as well as a repeating cystein-rich region, where intrachain disulfide binding occurs. Taken from Alberts et al. (2002)[52].

The binding of integrins to their ligands depends on extracellular divalent cations (Ca^{2+} or Mg^{2+}, depending on the integrin), reflecting the presence of divalent-cation-binding domains in the extracellular part of the α and β subunits. The type of divalent cation can influence both the affinity and the specificity of the binding of an integrin to its ligands. Most integrins are connected to bundles of actin filaments. After the binding of a typical integrin to its ligand in the matrix, the cytoplasmic tail of the β subunit binds to several intracellular anchor proteins, including α-actinin, talin, and filamin. These anchor proteins can bind directly to actin or to other anchorn proteins such as vinculin, thereby linking the integrin to actin filaments in the cell cortex[52]. Integrin expression is, therefore, vital to the migration of fibroblasts and other cell types.

The orientation of fibers in the matrix also influences cellular migration, in that cells tend to migrate along fibers and not across them. The ability of fibroblasts to migrate may be impeded by residual debris in the wound environment. To facilitate migration through such debris, fibroblasts secrete several proteolytic enzymes including MMP-1, gelatinase (MMP-2), and stromelysin (MMP-3). TGF-β stimulates fibroblasts to secrete these enzymes. The MMPs constitute a multigene family of over 25 secreted and cell surface associated or transmembrane enzymes. All MMPs are produced as zymogens containing a secretory signal sequence and a propeptide whose proteolytic cleavage is required for MMP activation. The propeptide is followed by the catalytic domain that contains the zinc binding motif. At least two MMPs (MMP-7 and MMP-26) are composed only of the signal peptide, propeptide, and catalytic domain, and are known as minimal domain MMPs (Figure 10). Most of the remaining MMPs contain a haemopexin-like domain that is thought to confer some degree of substrate specificity, and several have additional features, such as serine protease recognition motifs or fibronectin-like repeats (Figure 10). Finally, a subclass of MMPs contains a transmembrane and intracellular domain and are often referred to as MT-MMPs[53]. MMPs targets include other proteinases, proteinase inhibitors, chemotactic molecules, clotting factors, latent growth factors, growth factor-binding proteins, cell surface receptors, cell-cell adhesion molecules, and almost all structural extracellular matrix proteins. Thus MMPs are able to regulate many biologic processes and their activity is firmly regulated. MMPs are regulated at the transcriptional and post-transcriptional

levels and are also controlled at the protein level via their activators, their inhibitors, and their cell surface localization[54].

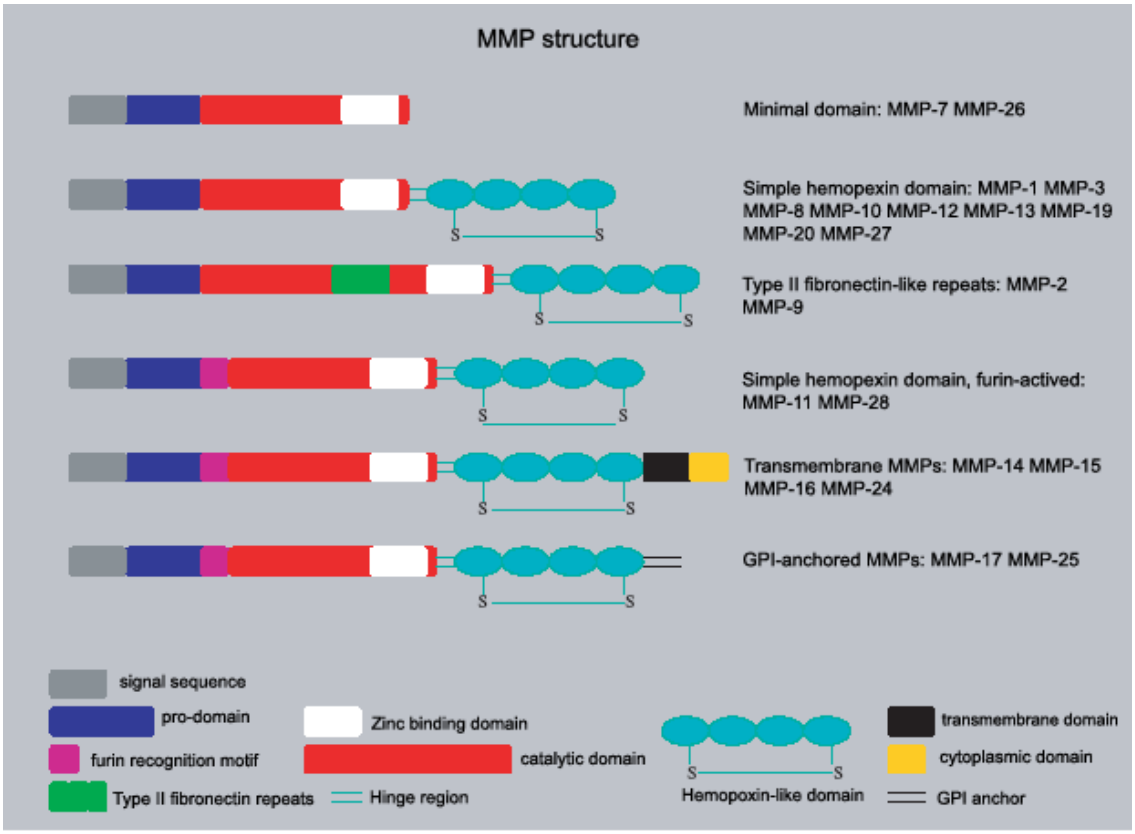


Figure 10. Protein structure of MMPs. The principal structural subclasses of MMPs are shown and the different domains indicated. Individual MMPs that belong to each structural subclass are listed. Taken from Stamenkovic (2003)[53].

Angiogenesis

During angiogenesis, endothelial sprouts derive from intact capillaries at the wound periphery. These sprouts grow through cellular migration and proliferation. Endothelial cells migrate during angiogenesis by forming transient contacts between their cells surface expressed integrins to the provisional fibrin-fibronectin matrix in a similar

manner as fibroblasts do. Upregulation of $\alpha_v\beta_3$ integrins is specifically associated with angiogenesis. Endothelial cell migration is also facilitated by the cell's ability to produce MMPs that break down collagen, fibronectin, laminins, elastin, and other extracellular matrix molecules. These breakdown products induce further induction of wound healing and tissue regeneration process. In addition, matrix degeneration permits endothelial cells movement and process formation. Angiogenesis process is regulated by a variety of growth factors and cytokines. The two most important growth factors that contribute to angiogenesis are vascular endothelial growth factors (VEGFs) and FGF-2. FGF-2 has a strong mitogenic effect on endothelial cells and promote endothelial cell proliferation and differentiation. They promote endothelial cell migration during the early phase of the wound repair through upregulation of the urokinase-type plasminogen activtor[55], which leads to clot formation and fibrin deposition, and facilitates the migration of endothelial cells through the fibrin clot. During granulation tissue formation, FGF-2 also promotes endothelial cell migration by induction of endothelial cell-surface $\alpha_V\beta_3$ integrin expression, which mediates the binding of endothelial cells to the extracellular matrix. VEGF is a potent mitogen for human microvascular endothelial cells and induces endothelial cell migration and sprouting by up-regulation of several endothelial integrin receptors including, $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_V\beta_3$. VEGF acts as a survival factor for endothelial cells through induction of the expression of the anti-apoptotic protein Bcl-2[56]. This pro-survival activity of VEGF requires the phosphatidylinositol 3 (PI3)-kinase/Akt signal transduction pathway[57].

Re-epithelialization

The processes of cellular migration and proliferation occur under the control of various growth factors including EGF, platelet-derived EGF, keratinocyte growth factor (KGF), and TGF-α. Some are produced by inflammatory cells and others are derived from the epithelial cells themselves. Epithelial cell migration requires the development of actin filaments within the cytoplasm of migratory cells and the disappearance of desmosomes and hemidesmosomes that link them to each other and to the basement membrane, respectively. At least some of these processes are dependent on changes in the integrin expression pattern on the cell membranes. For different integrins it has been shown that

they are up-regulated as soon as a certain stimulus occurs (e.g. VEGF-A165 and VEGF-R2). If the epidermal basement membrane is intact, cells simply migrate over it. In wounds in which it has been destroyed, the cells initially begin to migrate over and into the fibrin-fibronectin provisional matrix. As they migrate across the matrix, however, epithelial cells regenerate a new basement membrane. Re-establishment of a basement membrane under migrating cells involves the secretion of vitronectin, tenasin, and type I and V collagens. When epithelial cells reach each other from peripheral growth towards the middle of the wound they become contact inhibited and hemidesmosomes re-form between the cells and the basement membrane, and vitronectin and tenasin secretion decreases. A new functional basement membrane is formed and provides epithelial support as well as protection against the other milieu.

Protein synthesis and wound contraction

Collagen constitutes more than 50 % of the protein in scar tissue, and its production is essential to the healing process. Fibroblasts are responsible for the synthesis of collagen and other proteins synthesized during the repair process. The concentration of collagen subtypes varies among different tissues. Type I collagen predominates and forms 80 % to 90 % of the collagen found in the intact dermis. The remaining 10 % to 20 % is type III collagen. In contrast, granulation tissue that forms soon after the injury contains 30 % type III collagen. Accelerated type III collagen synthesis is correlated with fibronectin secretion after the injury. Type II collagen is found almost exclusively in cartilage, whereas type IV collagen is found in all basement membranes. Type V collagen is found in blood vessels, whereas type VII collagen forms the anchoring fibrils of epidermal basement membrane. Proteoglycans are also matrix components that are synthesized by fibroblasts after the injury. Their concentration in injured tissues gradually increases with time in a manner paralleling collagen synthesis. Proteoglycans consist of a protein core covalently linked to one or more glycosaminoglycans. Proteoglycans bind proteins and change their orientation in a manner that influences their activity. Dermatan sulfate is a proteoglycan that orients collagen molecules in a manner that facilitates fibril formation. Wound contraction is characterized by a predominance of myofibroblasts at the wound periphery. Although Gabbiani et al[58]

postulated that these cells were the 'motor' that contracted a wound. More recent work with collagen lattices has suggested that fibroblasts in the central part of the wound may be more important to the contraction process[48]. It is clear, however, that the process of wound contraction is cell mediated and does not require collagen synthesis. TGF-β and possibly other cytokines are involved in the wound contraction process[59].

Remodelling of the wound matrix

The nature of the wound matrix changes with scar tissue remodelling. Immature scar tissue contains a disorganized array of fine collagen fibers, which is gradually replaced by thicker fibers arranged in a parallel manner. In addition, the number of cross-links both within and between molecules gradually increases therefore slowly increasing the mechanical stability. As the nature of the collagen matrix changes, it becomes less cellular through apoptosis of cells involved in the healing process. The rate of collagen synthesis decrease and reaches an equilibrium with the rate of collagen breakdown. The downregulation of collagen synthesis is mediated by TNF-α[60], γ-interferon[61], and the collagen matrix itself[62]. MMPs are closely involved with the breakdown of collagen molecules that occurs actively during the remodelling process. Although scar tissue remodelling does not seem to be as complex as other aspects of the healing process, it is essential to the formation of a stable wound coverage leading to the formation of the mature scar tissue and rearrangement for tissue regeneration. Moreover, the remodelling process is associated with a substantial increase in wound-breaking strength[48].

The extracellular matrix as a scaffold for tissue reconstruction

The extracellular matrix is a complex mixture of structural and functional proteins, glycoproteins, and proteoglycans arranged in a unique, tissue specific three-dimensional ultrastructure. These proteins provide many functions including support and tensile strength, attachment sites for integrin cell surface receptors, and serve as a reservoir for signalling molecules and growth factors that modulate such diverse processes as cell migration, cell proliferation and orientation, angiogenesis and vasculogenesis, inflammation, immune responsiveness and wound healing. Stated differently, the ECM is a essential and dynamic component of all tissues and organs and is nature's natural

scaffold for tissue and organ morphogenesis, maintenance, and reconstruction following an injury.

Scaffolds for tissue reconstruction and replacement must have both appropriate structural and functional properties. However, the difference between structural and functional characteristics of the protein scaffolds is becoming increasingly blurred. Domains of proteins originally thought to have purely structural properties have been identified and found to have significant and highly modulating effects on cellular behaviour. For example, the RGD (arginine-glycine-aspartic acid) peptide that promotes adhesion of numerous cell types was first identified in the 10th fibronectin type III domain of fibronectin; a molecule originally described for its structural properties. Several other peptides have since been identified as 'dual function' proteins including types I and VI collagen, laminins, entactin, fibrinogen, and vitronectin. If one considers the ECM to be a degradable bioscaffold usable for implantation, both the structural and the functional components are transient due to the rapid rate of degradation of ECM scaffolds in vivo. Collagen is the most abundant protein within the ECM. More that 20 distinct types of collagen have been identified. The primary structural collagen in mammalian tissues is type I collagen. Collagen has maintained a highly conserved amino acid sequence through the course of evolution. For this reason allogeneic and xenogeneic sources of type I collagen have been long recognized as a useful scaffold for tissue repair with low antigenic potential. Collagen types other than type I exist in naturally occurring ECM, although in much lower quantities. Another example is type IV collagen being present within the basement membrane of all vascular structures. It is an important ligand for endothelial cells. Type VII collagen is an important component of the anchoring fibrils of keratinocytes to the underlying basement membrane of the epidermis. Type VI collagen functions as a 'connection' of functional proteins and glycosaminoglycans to larger structural proteins such as type I collagen, helping to provide a gel like consistency to the ECM. Type III collagen exists within selected submucosal ECMs, such as the submucosal ECM of the urinary bladder, where less rigid structures are required for its appropriate function. This diversity of collagens within a single scaffold material is partially responsible for the distinctive biologic activity of ECM scaffolds and is exemplary of the difficulty in designing such a

composite in vitro. In summary, the ECM is a rich source of numerous types of collagen and the relative concentrations and orientation of these collagens to each other provide an environment for cell growth both *in vitro* and *in vivo*.

Fibronectin, one of the 'dual function' proteins mentioned earlier, represents an important component of the ECM. Fibronectin is the second frequent molecule within the ECM. Fibronectin exists both in soluble and tissue isoforms and possesses many desirable properties of a tissue repair scaffold including ligands for adhesion of many cell types. Fibronectin exists in two-dimensional ECMs such as basement membranes as well as in three-dimensional ECMs such as submucosal structures.

Laminins are a family of complex adhesion proteins found in the ECM; especially within basement membrane ECMs. The prominent role of laminins in the formation and maintenance of vascular structures is especially noteworthy when considering the ECM as a scaffold for tissue repair. Vascularization of scaffolds for tissue repair is one of the rate limiting steps in the field of tissue engineering and proteins such as laminins are receiving much attention as an important component of endothelial cell friendly scaffold materials.

Glycosaminoglycans (GAGs) are important components of ECM and play important roles in binding of growth factors and cytokines, water retention, and maintaining the gel properties of the ECM. The heparin binding properties of numerous cell surface receptors and of many growth factors (e.g. FGF family, VEGF) make the heparin-rich GAGs extremely desirable components of scaffolds for tissue repair.

Although cytokines and growth factors are present within the ECM in small quantities, they act as potent modulators of cell behaviour. The list of growth factors is extensive and includes EGF, TGF-β, VEGFs, KGF, PDGF, and hepatocyte growth factor (HGF). These factors tend to exist in multiple isoforms, each with its specific biological activity. Purified forms of growth factors and biologic peptides have been investigated in recent years as therapeutic tools to encourage blood vessel formation (VEGF), inhibiting blood vessel formation (angiostatin), stimulating deposition of granulation tissue (PDGF), and encouraging epithelialization of wounds (KGF). However, these therapeutic approaches have struggled with determination of optimal dose, localized and

sustained release at the desired site, and the inability to turn the factors 'on' and 'off' as needed during the course of tissue repair[63].

Cell-ECM interactions

For tissue engineering strategies it is essential to know how cells can interact with the ECM and transmit the information received by the extracellular molecules into an intracellular event. Cell-matrix contacts have several major purposes: to anchor elements of the ECM to the cell surface; to form a continuous physical linkage between ECM and elements of the cytoskeleton that is required for cell adhesion and movement; to act as localised sites for transmission of mechanical force and elastic recoil between cells and ECM, and to act as sites for localised activity of signalling molecules. They are thus essential components in the integration and organisation of cellular and acellular elements within tissues. The principal structural elements of a cell-matrix contact are shown in Figure 11. On the extracellular face, matrix macromolecules bind to specific adhesion receptors, such as integrins, which are typically transmembrane or glycosylphosphatidylinositol-linked glycoproteins (see also Figure 9). On the intracellular side of the plasma membrane, the cytoplasmic domains of receptor molecules interact with either (i) cytoplasmic proteins which provide linkage to cytoskeletal filaments, or (ii) larger sets of cytoplasmic proteins which cluster by protein-protein interactions to form submembranous junctional complexes which then interface with cytoskeletal filaments[64]. The formation of molecular clusters at the cell surface lead to a direct or indirect control of cellular activities such as adhesion, differentiation, migration, proliferation and apoptosis, and are therefore essential in processes such as healing of tissue injuries or the progression of human cancer.

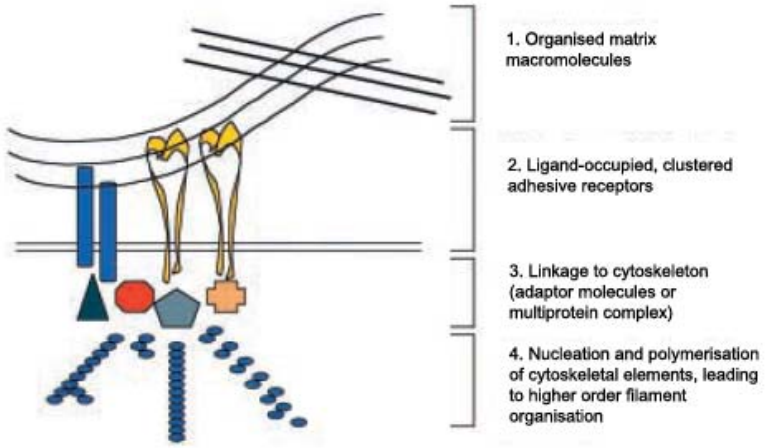


Figure 11. Schematic representation of the general structure of cell-to-matrix contacts. Not to scale. Taken from Adams (2001)[64].

Mechanotransduction at cell-matrix and cell-cell contacts

Cell-matrix adhesions depend on the differentiation state of cells, their physical location and the local forces sensed by the cells. One of the main challenges to understand cell-matrix adhesions is the enormous diversity and complexity of the in vivo microenvironments that cells encounter[65]. Recent studies have shown that many distinct types of adhesions exist between cells and the ECM; these adhesions differ in size, shape, and biochemical composition and probably differ in function as well[66]. Because these adhesions continually remodel in response to changes in the composition, architecture, and mechanical properties of the cell-matrix interface, they appear to be a central mechanism by which cells can change behaviour in response to structural and mechanical cues. The largest and most stable types of contacts include focal contacts or adhesion plaques, focal adhesions, fibrilar adhesions and hemidesmosomes. Other contact which are smaller, transient, or restricted distributed include filopodia, spikes, lamellae, podosomes and pseudopodia[64]. The best-characterized and largest of these structures is the focal adhesion (FA). FA complexes contain specific integrins (e.g. $\alpha_5\beta_1$ binding to fibronectin and $\alpha_v\beta_3$ linking to vitronectin) and a number of cytoskeleton-

associated proteins, such as talin, vinculin, α-actinin, filamin, and focal adhesion kinase, that connect the cytoplasmic tails of integrins with F-actin. These molecules form a physical bridge that spans from the ECM to the cytoskeleton. It is now generally accepted that mechanical forces can be transmitted across the membrane through integrins that mediate cell-ECM adhesion. For example, mechanical stresses applied via integrins resulted in coordinated changes in the cytoskeleton and the nuclear shape, indicating a long-range direct force-transmitting pathway from the cell surface into the nucleus[67]. Thus cell-matrix adhesions not only play a physical role in organizing cells into tissues but also provide an important biochemical role in the regulation of many cellular processes. Many signalling proteins (e.g., src, FAK, Ras) localize within cell-matrix adhesions. Because these signalling proteins function in cascades initiated by growth factors, their concentration at adhesions suggests that adhesions may act to coordinate integrin and growth factor signalling. Specific integrins within these adhesions appear to interact with specific growth factor receptors, these adhesions may enable cells to generate unique responses when exposed to particular combinations of ECM and growth factors. For example, signalling through basic fibroblast growth factor receptor in endothelial cells requires ligation of the integrin $\alpha_v\beta_3$, whereas signalling through vascular endothelial growth factor receptor uses $\alpha_v\beta_5$; because $\alpha_v\beta_3$ and $\alpha_v\beta_5$ bind to different classes of ECM proteins, the response of endothelial cells to a given growth factor depends on its underlying ECM. Thus adhesions provide a physical structure that allows many important biochemical signals to initiate fundamental changes in cell behaviour. Cell-cell adhesions, similar to cell-matrix adhesions, are emerging as important players in mechanotransduction. Distinct types of cell-cell adhesions include adherens junctions (AJs), tight junctions, and gap junctions, which are mediated by cadherins, occludins, and connexins, respectively. In some cases, especially with blood-borne cells, intercellular adhesions may be mediated by integrins. Among these different cell-cell adhesions, the AJ is perhaps one of the most important for transmitting mechanical signals directly to the actin cytoskeleton. The homotypic engagement of cadherins, a family of transmembrane Ca^{2+}-dependent adhesion molecules, initiates the formation of AJs and recruits scaffolding proteins that anchor the actin cytoskeleton. FAs and AJs exhibit many remarkable similarities. First, both

comprise of dense clusters of transmembrane receptors that attach the cell to the external environment. Second, both provide a highly dynamic and responsive mechanical link to the actin cytoskeleton. Third, the architecture of both FAs and AJ comprise of a large number of structural and signalling molecules that cluster at the junction through multiple, redundant protein-protein interactions. Several of these components, including α-actinin, vinculin, zyxin, moesin, and Arp2/3, are shared by both types of adhesions[68].

This work, set in the field of experimental tissue engineering, is motivated by the desperate request for a practical treatment to seal/heal PPROM. Presently, the therapeutic options to close a wound in premature ruptured fetal membranes are extremely limited. It is fair to say that there is no treatment of PPROM. The worthwhile consequence of an effective treatment of the ruptured fetal membranes could be to save the life and health of a baby and its mother. A particular challenge in our approach to treatment of PPROM is given to design a tissue engineered construct that can be delivered to the site of membrane rupture by minimal invasive methodology, namely fetoscopic methods. Our approach derived from the hypothesis that an amnion cell-matrix construct could permit an immediate closure of the amnion, therefore stopping the fluid leakage and promoting the wound closure in the fetal membranes. An additional aspect was to determine whether amnion cells derived from different gestational ages bear an inherent potential to proliferate, migrate and potentially heal a given injury.

Scope of the thesis

The aim of this thesis was to develop a tentative option for the treatment of preterm premature rupture of the membranes (PPROM) patients based on tissue engineering. The idea was to develop a cell-matrix system consisting of extracellular matrices colonized with human amnion epithelial and mesenchymal cells. Such cell-matrix systems may be precisely administered to the rupture site via endoscopical methods. In the recent years tissue engineering therapeutics approaches have become an important medical option after injury in many aspects of modern medicine.

Three major aims were formulated:

First: human amnion cell isolates were generated as a cellular source for the reconstruction of a tissue engineered amnion membrane after PPROM, and the establishment of conditions for their expansion *in vitro* were characterized **(Chapter 2)**. The proliferation potential of cultured human amnion epithelial and mesenchymal cells from preterm and term placenta was investigated under various cell growth conditions.

Secondly: the repair potential of human amnion cells from preterm and term placenta was examined and compared **(Chapter 3**). Repair potential and effect of potential proliferation stimulants were investigated using an *in vitro* lesion repair assay.

Finally: the most critical and challenging part of this project involved the design of a cell containing matrix that mimics the architecture of the native amnion, described in **Chapter 4**. The behaviour of human amnion cells in three-dimensional (3D) collagen I and fibrin matrices was investigated. The envisioned aim was to develop a defined cell-containing piece of matrix that can potentially be used to seal/heal the amnion membrane after PPROM *in vivo*.

Chapter 2

Inducing proliferation of human amnion epithelial and mesenchymal cells for prospective engineering of membrane repair

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Abstract

Objective: To prepare a tissue engineering approach to fetal membrane repair after premature rupture of the membranes (PROM) by characterizing the proliferation potential of human amnion epithelial and mesenchymal cells from preterm and term placenta in primary culture.

Methods: Amnion epithelial and mesenchymal cells from 15 preterm (23-36 week) and 27 term placentas collected at cesarean section were separated enzymatically, characterized immunohistochemically (anti-cytokeratin-18 and anti-E-cadherin, and anti-vimentin, respectively), and their ratio determined. Proliferation on tissue culture polystyrene (TCPS) or collagen in one medium and on TCPS in four different media after 14 days was measured photometrically and compared in preterm vs. term placenta. For statistical analysis the Mann-Whitney test was used.

Results: Preterm and term epithelial : mesenchymal cell ratios were 4.3:1 and 7.8:1. Term epithelial cells proliferated similarly on TCPS or collagen. Mesenchymal cells proliferated only with fetal bovine serum (FBS). Proliferation of term amnion cells in medium containing FBS, epithelial growth factor (EGF), insulin, transferrin and triidothyronine (T3) was significantly increased ($p<0.001$) compared with the other three media, and percentage proliferation was slightly higher in preterm cells.

Conclusion: Characterization of human amnion epithelial and mesenchymal cells identified the most potent proliferation-inducing medium yet. Studies of the wound-healing potential of these cells are needed, examining their behavior and proliferation on fibrin microbeads and other extracellular matrixes as the next step towards engineering membrane repair in PROM.

Keyword: Cell culture conditions, epithelial cells, growth factors, human amnion, medium, mesenchymal cells.

Supported by the EMDO Foundation Zurich, Switzerland.

Introduction

Spontaneous premature rupture of membranes (PROM) complicates about 1 % of all pregnancies and is the leading cause of perinatal morbidity and mortality[69, 70], especially in the previable period[70]. Iatrogenic PROM is a major limitation of uterine endoscopy; the incidence is 10 % after laser coagulation for twin-twin transfusion syndrome[17], >30 % after fetoscopic cord ligation[18], and >62 % after endoscopic tracheal clipping[19]. Most research has concentrated on the pathogenesis of PROM[71, 72] while largely neglecting the repair and healing potential of fetal membrane. The association of changes in the secretion of matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors (TIMPs) with the restoration of fetal membrane integrity in an animal model following fetoscopy suggests an active repair process[73]. Clinical experience also supports the hypothesis of fetal membrane repair given the very low incidence of PROM after routine midtrimester amniocentesis[74, 75] and the spontaneous 'resealing' reported in 7.7 % to 9.7 % cases of PROM[24, 76].

Several treatments for PROM have been described, from collagen plugs in a rabbit model[41] to amniopatch[30], amniograft[46], maternal blood clot patch[29] and fibrin glue[77] in humans. However, cell-based or tissue-engineered therapies, e.g. using cartilage[78], endothelium[79] or skin[80], have since become an alternative and promising option after injury in many branches of medicine. To develop a new approach to restoring the fetal membrane integrity after PROM, we therefore wished to characterize and compare the proliferation potential of cultured human amnion epithelial and mesenchymal cells from preterm and term placenta under a variety of growth factor conditions.

Material and methods

Primary human amniotic cell cultures

15 preterm (23-36 week) and 27 term (37-41 week) placentas with attached membranes from women without PROM, signs of infection or fetal chromosomal abnormalities were collected immediately after elective cesarean section. Amnion and chorion laeve tissues were separated by blunt dissection[81], the amnion was cut approximately 2 cm from the placental disc to avoid the 'zone of extreme altered morphology'[82] and then washed in phosphate buffered saline (PBS; Sigma-Aldrich, Switzerland) to remove cellular debris and blood. Amnion epithelial and mesenchymal cells were separated by differential enzymatic dissociation[6] and their ratio determined. Cell viability assessed by trypan blue dye exclusion was 97 %. After plating at a density of 5000 per 50 µl medium per well (96-well MicroWell® plates, Nunc Nunclon), the cells were maintained in culture in a humidified atmosphere at 37° C and 5 % CO_2 with medium replacement after 48 or 72 h.

Cell characterization by immunohistochemistry

The cells were characterized using antibodies specific for two separate filament proteins expressed in epithelial and mesenchymal cells, respectively. The epithelial cell line marker, cytokeratin-18, was detected by a monoclonal antibody (Sigma-Aldrich, Switzerland) at 1:1000 dilution. The mesenchymal cell line marker, vimentin, was detected by a monoclonal antibody (Sigma-Aldrich, Switzerland) at 1:100 dilution. Epithelial cells were also identified using, at 20 µg/ml, a monoclonal antibody (R&D

Systems, Europe) which detects E-cadherin[83], the cell adhesion molecule specific to human epithelium.

Antibodies were diluted in 2 % bovine serum albumin (BSA) in PBS. Secondary antibodies were conjugated to tetramethylrhodamine isothiocyanate (TRITC; Sigma-Aldrich, Switzerland) or fluorescein isothiocyanate (FITC; Sigma-Aldrich, Switzerland). Anti-mouse IgG labelled with TRITC was used at 1:200 dilution and anti-goat IgG labeled with FITC at 1:400 dilution.

Cells were fixed and permeabilized with ethanol (-20° C) before being rinsed with PBS. They were then incubated with 2 % BSA in PBS to block nonspecific binding. Primary antibodies were applied and the cells incubated at room temperature for 1 h, then washed three times with PBS and incubated in the dark with second antibody at room temperature for 1 h. After the final PBS wash, they were analyzed by fluorescence microscopy (Carl Zeiss) at ×20. Control cells were treated identically without the primary antibodies. Cell images (CCD camera, Hamamatsu) were processed using QWin software (Leica Microsystems) and Photoshop version 5.0.

Cell proliferation assay

Cell cultures were tested after 1, 3, 7 and 14 days of culture using the proliferation reagent WST-1 (Roche, Switzerland). The assay is based on the reduction of WST-1 to water-soluble formazan by viable cells[84]. After incubating the cells with WST-1 for 2 h, formation of the formazan dye in the microtiterplate was quantitated with an ELISA plate reader at 450 nm. Absorbance correlated directly with the number of viable cells. Experiments were done in duplicate and the means used for analysis.

Variation of culture conditions, gestational age and media

We varied the following factors in our proliferation parameter test system (Figure 1):

• culture conditions: cells from term placenta (n=5) were cultured on tissue culture polystyrene (TCPS) and collagen I (Sigma-Aldrich, Switzerland) using a serum-free medium (medium 1: Ham's F12:DMEM + 50 ng/ml EGF, 2.5 μg/ml insulin, 5 μg/ml transferrin, and 0.1 ng/ml T_3) shown to be optimal for human amnion epithelial cells[85] and containing known factors at precise concentrations.

• gestational age (GA): cells from preterm placenta (n=3) were cultured on either TCPS or collagen I using the same serum-free medium.

• media: cells from preterm (n=4) and term (n=18) placenta were tested in three other media (medium 2 = medium 1 + 50 ng/ml EGF; medium 3 = medium1 + 10 % FBS; medium 4 = Ham's F-12:DMEM (1:1) + 10 % FBS).

After 14 days we compared percentage proliferation between preterm and term cultures, defining proliferation as a fourfold increase over baseline (D1) absorbance (WST-1); this was on the basis of an amnion cell doubling time of 150-210 h, which translates into an approximate fourfold increase in cell count after 14 days[86]. Our hypothesis, based on the known increase in apoptosis with GA[87], was that preterm cultures would show the higher proliferation potential.

Statistical analysis

Results were presented as the mean ± SD. For calculation of significance ($p<0.05$) the Mann-Whitney test was used.

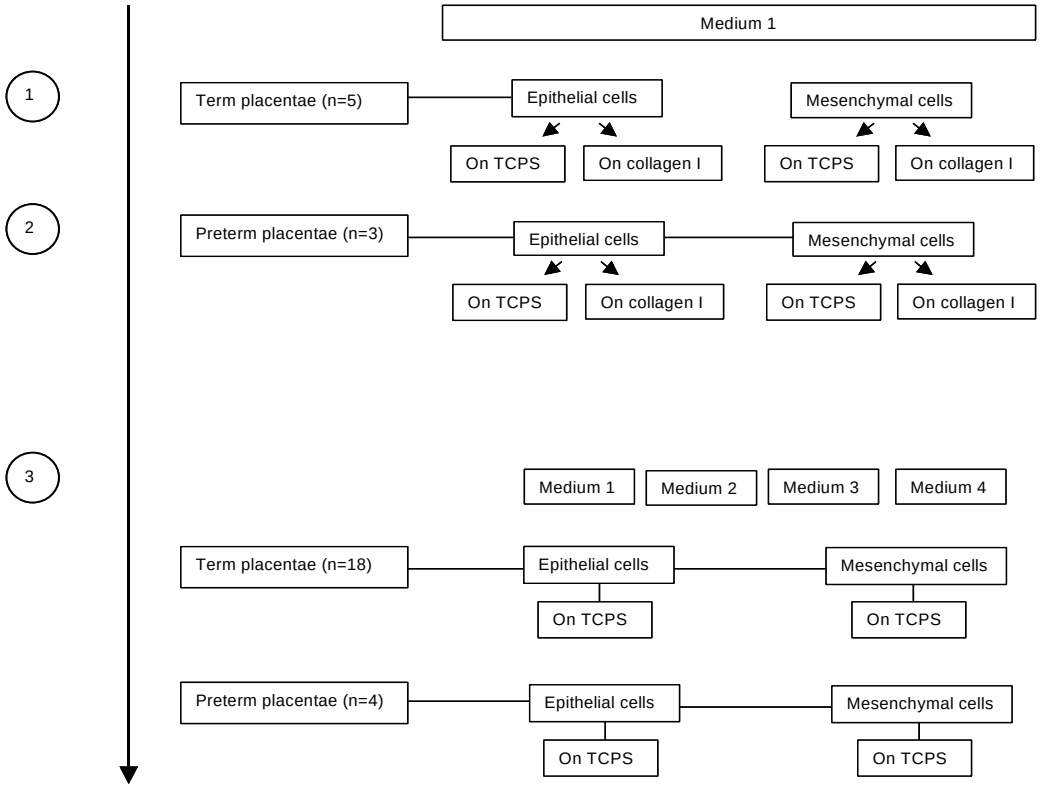


Figure 1. Study flowchart.

Results

Epithelial:mesenchymal cell ratios

The yield of amnion epithelial cells was approximately 2 million/g of amnion tissue. Epithelial : mesenchymal cell ratios, plotted vs. GA in Figure 2, were 4.3:1 and 7.8:1 in preterm and term placenta, respectively.

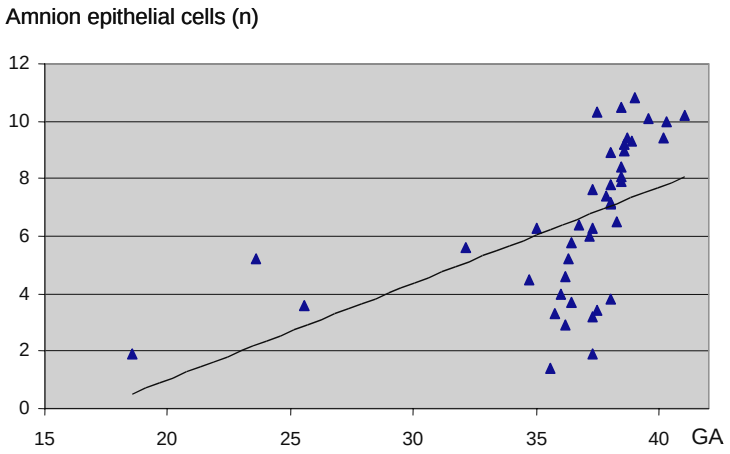


Figure 2. Amnion epithelial (n) : mesenchymal (1) cell ratio and gestational age (GA).

Identification and characterization of epithelial and mesenchymal cells

After three days in culture, cells isolated using trypsin digestion which stained positive for cytokeratin-18 and E-cadherin but negative for vimentin were identified as epithelial. Those isolated with collagenase treatment and staining positive for vimentin but negative for cytokeratin-18 and E-cadherin were identified as mesenchymal. In monolayer culture, epithelial cells appeared rounded and cuboidal, forming clusters

across the culture plate. Mesenchymal cells had a fusiform spindle shape with cytoplasmic pseudopodia.

Effect of culture conditions, media and gestational age on cell proliferation

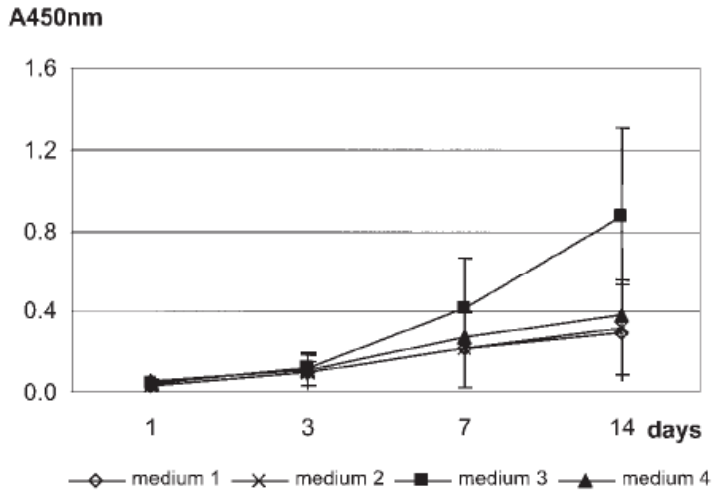
Having demonstrated that proliferation of term epithelial cells on TCPS using serum-free medium[85] was similar that on collagen I (0.633±0.414 vs. 0.648±0.448; p=0.83), we performed all further experiments on TCPS, for cost and time reasons, and because the harvest of term mesenchymal cells was low. Proliferation of epithelial cells of preterm vs. term placental origin cultured under similar conditions in medium 1 did not show a statistically significant difference (p=0.30). However, preterm and term mesenchymal cells failed to proliferate on either surface. We therefore tested term epithelial and mesenchymal cells in three other media in an attempt to optimize mesenchymal-biased proliferation. Epithelial cell growth patterns and morphology were similar in all four media. Term epithelial proliferation (Figure 3A) was similar in serum-free media (media 1+2) and 10 % FBS-containing medium (medium 4) and highest in medium 3.The same results were found for preterm epithelial cell proliferation (medium 1: 0.082±0.72; medium 2: 0.166±0.124; medium 3: 0.827±0.675; medium 4: 0.254±0.186). For term epithelial cell proliferation statistical analysis showed that proliferation in medium 3 was highly significantly increased ($p<0.001$) compared with cell proliferation in media 1, 2 and 4. However, for preterm epithelial cell proliferation no significant difference was found between medium 3 and 4 (p=0.15),

but proliferation in medium 3 tended to be increased compared with medium 2 and 1 ($p=0.08$).

In the case of mesenchymal cells, medium 3 also gave the highest proliferation for both preterm (medium 1: 0.183±0.121; medium 2: 0.228±0.120; medium 3: 1.081±0.668; medium 4: 0.913±0.337) and term (Figure 3B) cells, followed by medium 4, which contained only 10 % FBS. Serum-free media 1 and 2 failed to stimulate mesenchymal proliferation (Figure 3B). Statistical analysis showed that proliferation of term mesenchymal cells in medium 3 was highly significantly increased ($p\leq0.001$) compared with cell proliferation in media 1, 2 and 4; for preterm mesenchymal cell proliferation no statistical difference was detected between mediums 3 and 4 ($p=0.7$).

Concerning the percentage proliferation at day 14, with media 3 and 4 preterm epithelial and mesenchymal proliferation was similar to, or slightly higher than, its term counterpart (Figure 4).

A



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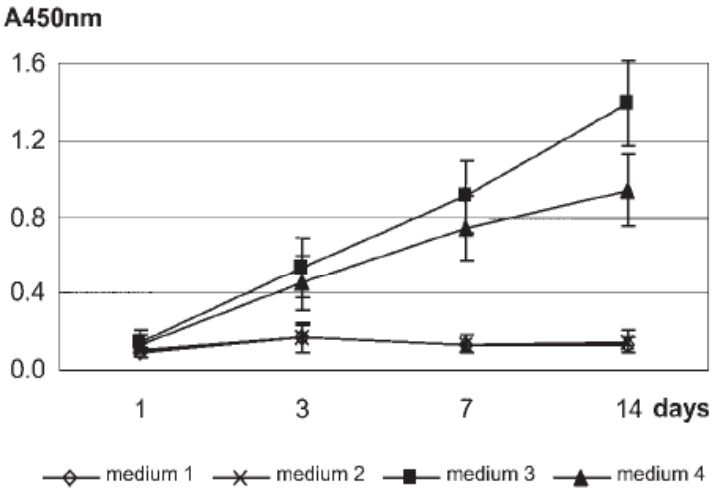


Figure 3. Proliferation of human amnion cells from term placenta (mean ± SD/2) in four different media (media 3 & 4 contained 10 % FBS): (A) Epithelial cells; (B) Mesenchymal cells.

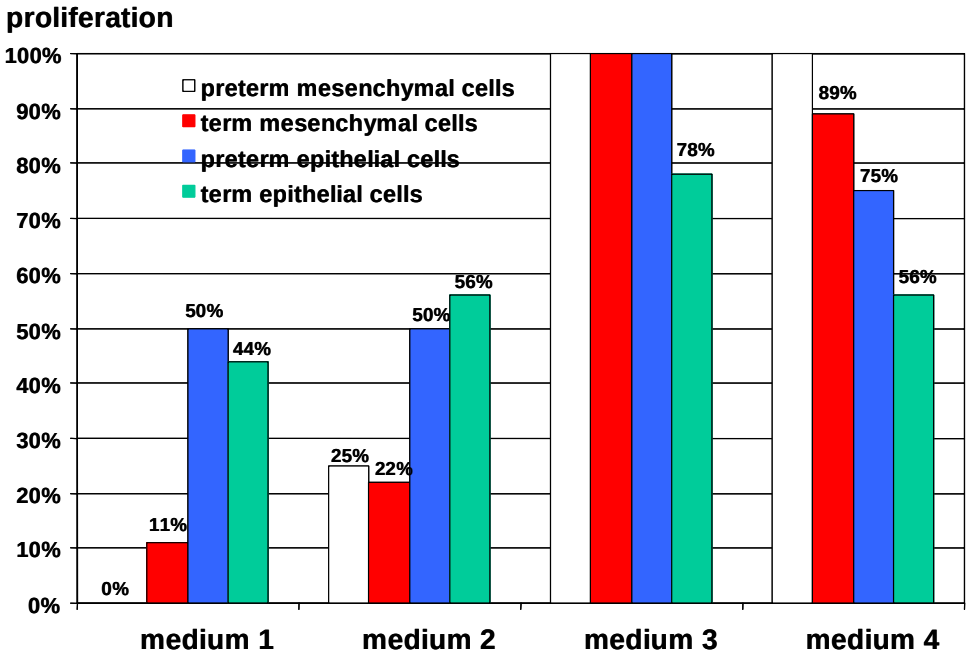


Figure 4. Proliferation (%) of amnion epithelial and mesenchymal cells from preterm vs. term placenta after 14 days culture.

Discussion

To our knowledge this is the first report on the effect of varying culture conditions and media (± serum, and with growth factors in different concentrations) on the growth and proliferation of human amnion epithelial and mesenchymal cells. Our medium 3 elicited significantly higher term epithelial and mesenchymal proliferation rates than culture media previously described for amnion cells (primarily DMEM + 10 % FBS). Mesenchymal cells failed to proliferate without FBS, i.e. FBS contains a factor, as yet unidentified, which is essential for the induction of mesenchymal proliferation.

The strengths of our study are that we included a large number (n=42) of human amnion cell cultures, analyzed mesenchymal in addition to epithelial cells, and examined GA-dependent proliferation. However, the proportion of preterm membrane cultures remained low as we had to exclude many cases with PROM: Garzetti et al.[88] described decreased cell growth potential in PROM amnion culture. For this reason, we could not attribute statistical significance to our finding, in line with our hypothesis based on GA-dependent apoptosis[87], that percentage proliferation was slightly higher in amnion cells from preterm *vs* term placenta. However, our result agrees with the findings of both Terada et al.[7], who reported that amnion cells harvested at elective cesarean section ceased to proliferate after 11 days in culture despite regular replacement of fresh medium and cytokines, and Casey and MacDonald[6], who found that the capacity for interstitial collagen synthesis was greatest in amnion from pre-16 week pregnancies, after which it rapidly declined.

A weakness of our study is that we tested cell proliferation only on TCPS and collagen I but not on other extracellular matrixes, some of whose proteins possess intrinsic growth factor or mitogenic activity[89]. The reason for using TCPS and the natural substrate, collagen, is that on starting our study we obtained just enough cells for two different experiments.

Few data are available in the literature on human amnion cell growth. Shortcomings in previous studies have included failure to specify the number of cell cultures examined[7, 85, 90] or the exact GA of the amnion of origin[7], thereby neglecting an important determinant of both viability and cell distribution: our study confirmed the histological evidence[91] for a disproportionate increase during gestation of epithelial over

mesenchymal cells. Other shortcomings have been the use of term placenta only[85, 90], analysis of epithelial cells only[7], and failure to isolate epithelial and mesenchymal cells[85, 90] - of particular importance given that cell preparations can vary enormously in epithelial:mesenchymal ratio, from 3:97 to 87:13[92].

Amnion cells in primary monolayer culture share several morphologic, biochemical, and enzymatic characteristics with cells in amnion tissue[81]. Culture is therefore a suitable technique for studying amnion cell proliferation with the long-term purpose of tissue repair in selected cases of PROM. We did not use amniotic fluid as a cell source for fetal tissue engineering[93] because of maternal cell contamination[94] and the presence of cells of different tissue origins[95].

The advantage of using heterologous human amnion cells for tissue repair is that amnion is readily and plentifully available. Human amnion epithelial cells also do not express the human leukocyte antigens (HLA)-A, -B, -C or -DR, suggesting that they do not induce immune rejection after allotranplantation[7]. The use of human heterologous amnion cells for engineered repair of PROM thus seems a promising strategy, after the ultimately unsuccessful experiments with alternative approaches, including amniopatch[30], amniograft[46], maternal blood clot patch[29] and fibrin glue[77]. Maybe biodegradeable fibrin microbeads (FMB) as potent cell carriers[96, 97] are useful to bring the amnion cells by injection to the rupture side and induce amnion cells to migrate into the whole and close it. In the pig skin dermal wound model FMB seemed to be very promising, as by day 3 only wounds which had been implanted with the combination of FBS + fibroblasts demonstrated accelerated healing and substantial formation of granulation tissue[96]. We are therefore planning further studies, in order to exam the

behavior and proliferation of human amnion cells on FMB and other extracellar matrixes and to evaluate the wound healing potential of human amnion cells.

In conclusion, we have characterized human amnion epithelial and mesenchymal cells in culture, induced cell proliferation with different media, and found one medium which stimulated proliferation better than previously published media. We intend to use these data to evaluate the wound healing potential of human amnion cells by examining their behaviour and proliferation on fibrin microbeads and other extracellular matrixes, with the ultimate aim of engineering the tissue repair of PROM.

Chapter 3

In vitro lesion repair by human amnion epithelial and mesenchymal cells

Published in: Am J Obstet Gynecol 2004;190:87-92.

Abstract

Objective: To compare 'wound healing' by human amnion epithelial and mesenchymal cells from preterm and term placenta using an *in vitro* lesion repair assay.

Study design: Lesions were created in confluent monolayers of amnion epithelial and mesenchymal cells from preterm and term placentas. Repair was monitored by measuring lesion area and the response to potential stimulants (platelet derived growth factor, tumor necrosis factor α, fibrinogen and phorbol myristate acetate). Cell proliferation was detected using 5-bromodeoxyuridine staining.

Results: Lesion repair was complete within 40 h in control epithelial cultures from preterm and term placenta but incomplete in mesenchymal cultures (preterm cells: 80%; term cells: 40%). Platelet derived growth factor, tumor necrosis factor α, fibrinogen, and phorbol myristate acetate did not accelerate repair in either cell type.

Conclusion: An *in vitro* lesion repair assay revealed differences in lesion repair capacity between amnion epithelial and mesenchymal cells and between mesenchymal cells from preterm and term placenta.

Key words: amnion epithelial and mesenchymal cells, preterm and term placenta, wound healing

Supported by the EMDO Foundation Zurich, Switzerland.

Introduction

Premature rupture of the membranes (PROM) occurs in approximately 1 % of all pregnancies and 30-40 % of preterm deliveries[71]. Both PROM and preterm birth carry a high risk of maternal morbidity and neonatal morbidity and mortality[98]. Several treatments for PROM in human fetal membrane models have been proposed, including amniopatch[30, 99, 100], amniograft[46], maternal blood clot patch[29] and fibrin glue[40, 77], but clinical studies have been few and success limited: none of these treatments have yet been introduced into clinical routine.

Most studies have focused on the pathogenesis of PROM[72, 101-103] but few have addressed the repair potential of the fetal membranes. Yet clinical experience supports the assumption that the fetal membranes are capable of self-repair: the incidence of PROM after routine midtrimester amniocentesis is very low[74, 75] and spontaneous resealing after PROM is reported in 7.7 % to 9.7 % of cases[24, 76]. The aim of the present study was to investigate and compare the repair potential of amnion epithelial and mesenchymal cells from preterm and term placenta using an *in vitro* lesion repair assay with the ultimate objective of designing therapeutic strategies for PROM.

Material and methods

Isolation and culture of amnion epithelial and mesenchymal cells

Amnion tissues were collected immediately after elective cesarean section from five preterm (32-36 week) and four term (37-38 week) placentas with attached membranes from women without PROM, signs of infection or chromosomal abnormalities. Amnion and chorion laeve tissues were separated by blunt dissection[81, 104], and the amnion was cut approximately 2 cm from the placental disc to avoid the 'zone of altered morphology'[82, 105]. The samples were washed in phosphate buffered saline (PBS; Sigma, Buchs, Switzerland) to remove cellular debris and blood, then minced and treated four times with 0.25 % trypsin (Gibco BRL, Basel, Switzerland) for 15 min. The first digestion supernatant, consisting primarily of red blood cells, was discarded. Epithelial cells obtained during subsequent trypsinizations were pelleted by centrifugation at 1000 rpm for 5 min and resuspended in a basal medium comprising a 1:1 mixture of Ham's F-12 and Dulbecco's minimal essential medium (Gibco) containing 10 % heat inactivated fetal bovine serum (FBS; Gibco), 100 U/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco). Mesenchymal cells were isolated from the remaining amnion tissue after complete removal of epithelial cells (evaluated microscopically). The tissue was minced and incubated with 2 mg/ml collagenase A (Roche Diagnostics, Rotkreuz, Switzerland) at 37° C for 120 min. Dispersed mesenchymal cells were collected by centrifugation at 1000 rpm for 5 min. Epithelial and mesenchymal cell viability assessed by trypan blue dye exclusion was 97 %. Both cell types were cultured further in basal medium supplemented with 50 ng/ml epidermal growth factor (EGF; Sigma), 2.5 μg/ml insulin (Sigma), 5 μg/ml transferrin (Sigma),

and 0.1 ng/ml triiodothyronine (T_3; Sigma) ['supplemented basal medium']. The cells were maintained in culture until confluence in a humidified atmosphere at 37° C and 5 % CO_2.

Microscopy

Samples were analyzed by phase contrast and fluorescence microscopy (Zeiss, Axiovert 135, Oberkochem, Germany). The images were taken by a Hamamatsu CCD camera and processed using LeicaQ Win Image Analysis software (Leica Imaging System, Ltd., Cambridge, England, UK) and Photoshop version 5.0. Experiments were done in duplicate, and four fields of each sample were analyzed.

***In vitro* lesion repair assay**

Cells were grown to confluence in 48-well plates (Nunclon delta, Nunc, D-65203 Wiesbaden) in supplemented basal medium. Prior to assay they were challenged overnight with serum-free medium. The monolayer was lesioned using a ~2 mm cell scraper without damaging the dish surface. Immediately post-lesion, parallel samples were incubated with either 100 ng/ml platelet-derived growth factor (PDGF; Sigma), 50 ng/ml tumor necrosis factor α (TNFα; Sigma), 3 mg/ml fibrinogen (Sigma), or 50 ng/ml phorbol myristate acetate (PMA; Sigma) Lesion areas were imaged at 0 h, 16 h and 40 h and, after manual analysis using image processing software, calculated by subtracting the area in μm^2 between the lesion edges from the area measured at 0 h. Values were expressed as a percentage of the 0 h area (100 %).

Cell proliferation assay

To detect and quantify proliferating cells, 5-bromo-2′-deoxyuridine (BrdU) incorporation into cellular DNA was determined according to the manufacturers' instructions (Sigma): 16 h post-lesion, the cells were incubated ± stimulating factors with 10 μM BrdU for 90 min at room temperature. They were then fixed in absolute methanol for 10 min at +4° C and air dried after removal. DNA was denatured by incubating the cells in 2 M HCl for 60 min at +37° C. The cells were further immersed in 0.1 M borate buffer, pH 8.5, to neutralize the acid. The buffer was changed twice in 10 min. The cells were then washed 3 times in 10 min with PBS before being placed in a humidified chamber and incubated with anti-bromodeoxyuridine-fluorescein [diluted 1:10 in PBS supplied with 0.1 % bovine serum albumin (BSA; Sigma)] overnight at 4°C. After repeated washing the cells were analyzed by fluorescence microscopy. BrdU incorporation was quantified by calculating the percentage of BrdU positive cells both in the perilesional area and at the cell culture edge furthest from the lesion.

Results

Repair of amnion epithelial and mesenchymal cells in monolayer cell cultures

Epithelial and mesenchymal cells differed in repair potential. The control epithelial cell monolayer from preterm placenta was fully repaired within 40 h (Figure 1A). The cells appeared rounded and cuboidal, similar to their pre-lesion appearance, i.e. their structural integrity was maintained. Addition of PDGF, TNFα, and fibrinogen had no stimulant effect (Figure 1C), while PMA markedly increased lesion area over the test period. Term placenta epithelial cells gave similar results (Figures 1B and D). In mesenchymal cells from preterm and term placenta, repair at 40 h was incomplete (80 % and 40 %, respectively; Figures 2A and B). Perilesional mesenchymal cells acquired a migratory phenotype with extension of lamellipodia and cytoplasmic protrusions in contrast to the spindle shaped cells within the confluent monolayer. Preterm mesenchymal cells had approximately twice the repair potential of their term counterparts; in neither case was repair stimulated by PDGF, TNFα, or fibrinogen (Figures 2C and D). PMA had the same effect as on preterm and term epithelial cells.

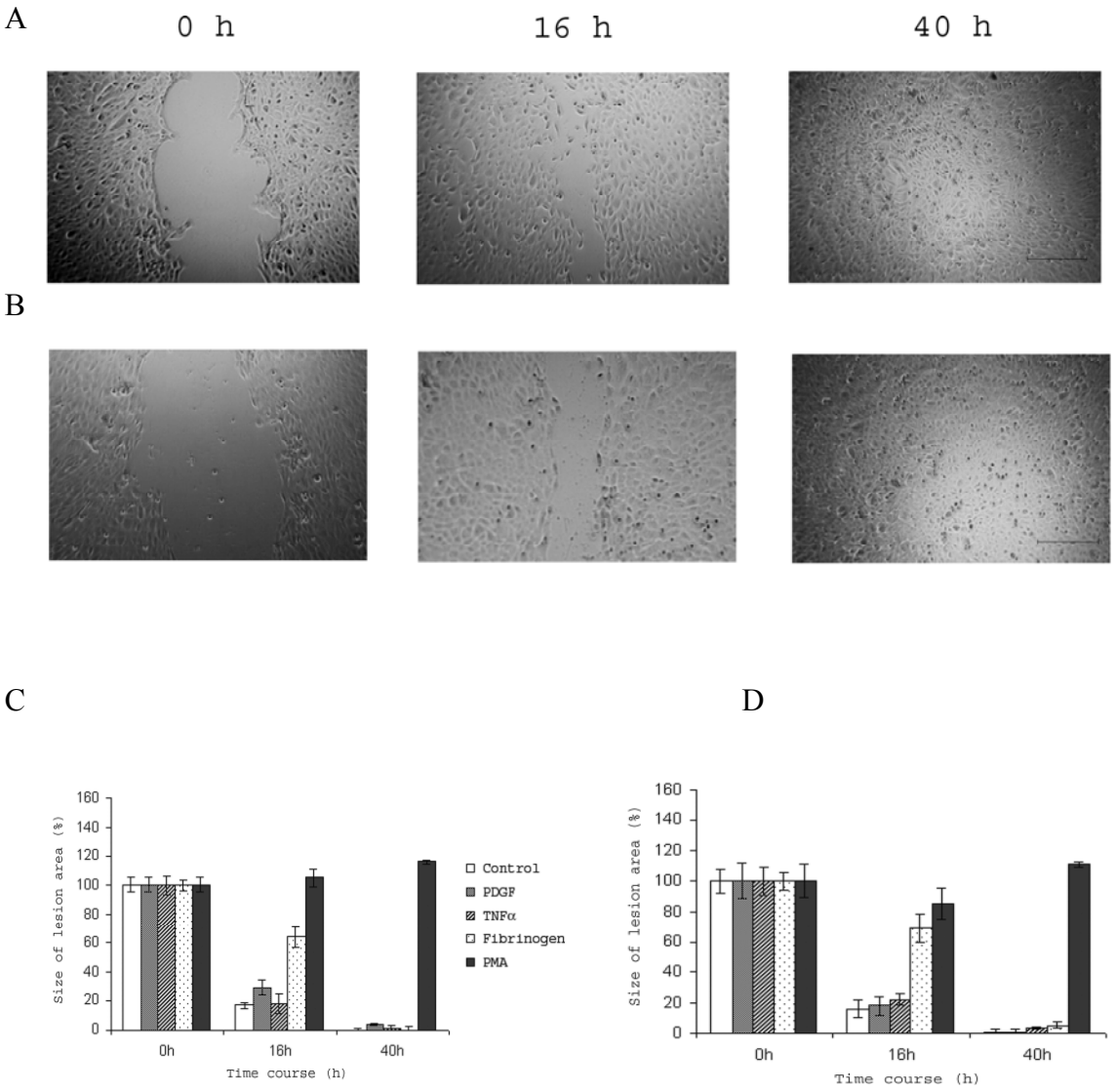


Figure 1. Timed phase contrast images of lesion areas (scale bar, 1 mm) in control amnion epithelial cells from preterm (A) and term (B) placenta show complete lesion repair within 40 hours. Timed lesion repair, expressed as the percentage of original lesion area (mean ± SD) of five preterm (C) and four term experiments (D), in control *(open bars)* and treated epithelial cultures show no acceleration in response to PDGF *(diamond hatched bars)*, TNFα *(hatched bars)*, fibrinogen *(dotted bars)*, and PMA *(closed bars)*.

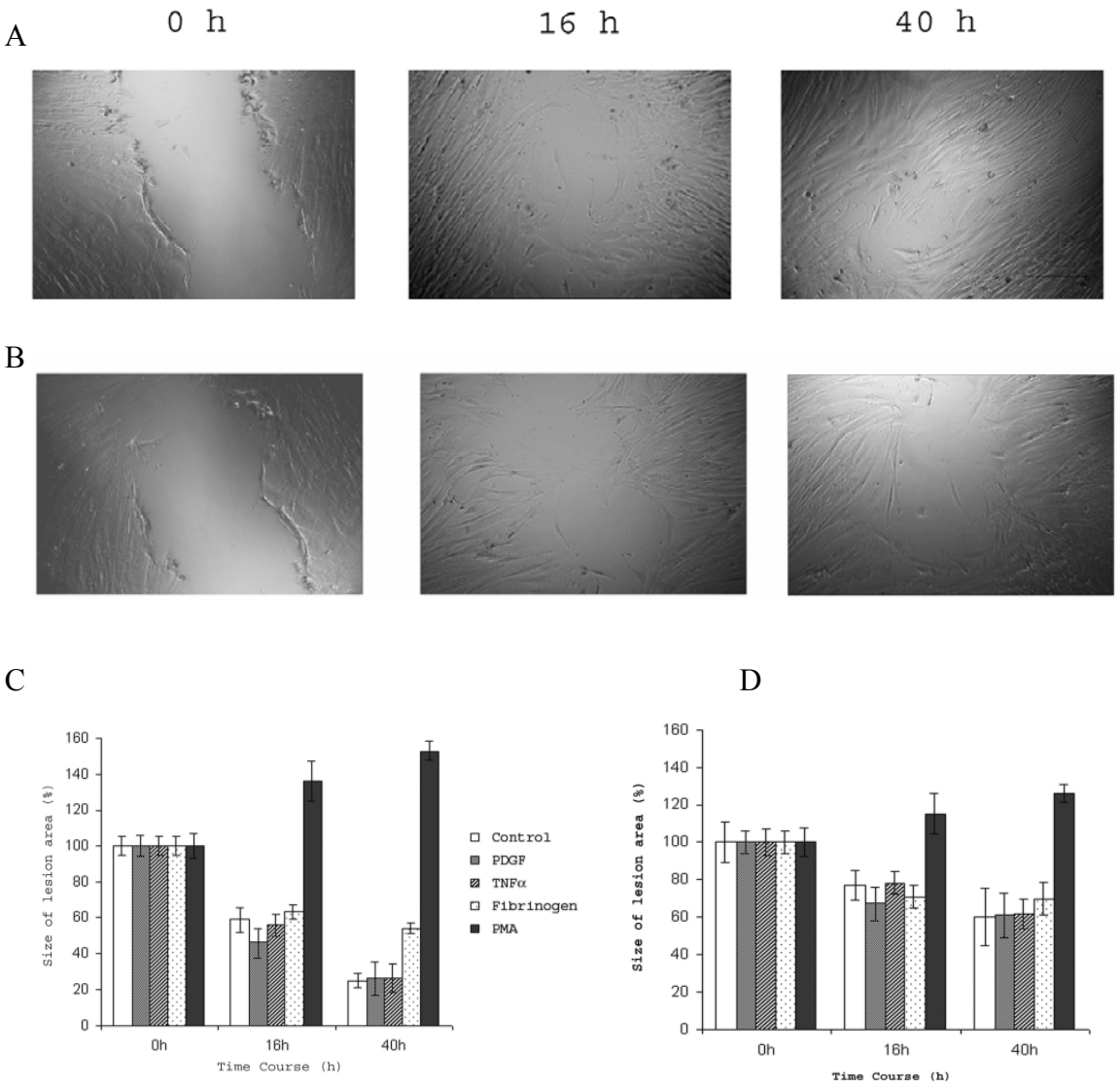
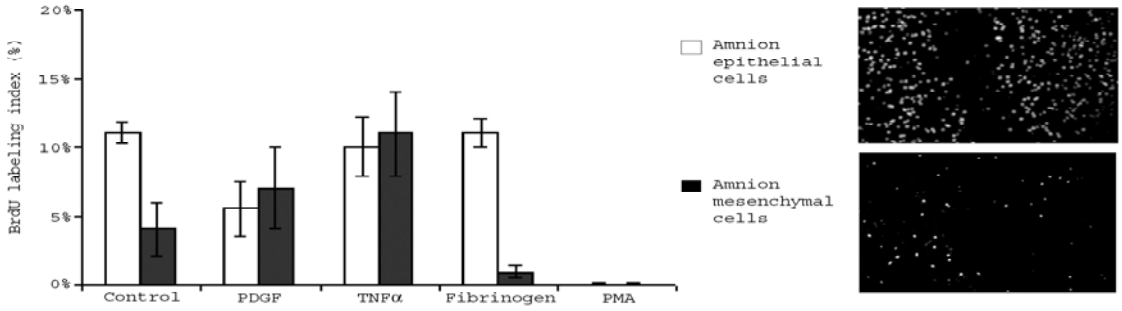


Figure 2. Timed phase contrast images of lesion areas (scale bar, 1 mm) in control amnion mesenchymal cells from preterm (A) and term (B) placenta show 80 % and 40 % repair, respectively, at 40 hours. Timed lesion repair, expressed as the percentage of original lesion area (mean ± SD) of five preterm (C) and four term (D) experiments in control (open bars) and treated mesenchymal cultures show an approximate 2-fold increase in lesion repair in preterm versus term cultures and no acceleration in response to PDGF *(diamond hatched ba*rs), TNFα *(hatched bars)*, fibrinogen *(dotted bars)*, and PMA *(closed bars)*.

Amnion epithelial and mesenchymal cell proliferation

In both epithelial and mesenchymal cells from preterm and term placenta, BrdU positive cells were detected in the perilesional area and at the culture edge (Figure 3A and B). PDGF, TNFα, and fibrinogen did not stimulate preterm epithelial proliferation (Figure 3A) versus control; PDGF was even inhibitory. In contrast, mesenchymal incorporation of BrdU increased versus control in the presence of PDGF (2.5 %) and especially TNFα (5 %). In fact TNFα increased preterm mesenchymal cell proliferation by 100 %. BrdU positive cells were rare after the addition of fibrinogen, and absent in both epithelial and mesenchymal cultures after PMA. Term epithelial cell proliferation was approximately 10% that of its preterm counterpart (control group; Figures 3A and B); it was similar to the control after the addition of PDGF, TNFα, fibrinogen and PMA (Figure 3B). BrdU incorporation by term mesenchymal cells was approximately 50 % greater (control group) than by their preterm counterparts (Figures 3A and B), and was not stimulated by any factor. However, both PDGF and TNFα increased proliferation in preterm mesenchymal cells.

A



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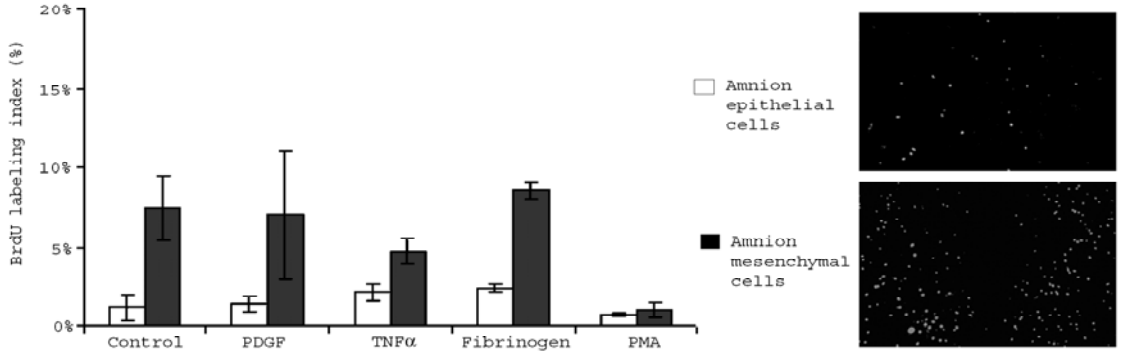

Figure 3. BrdU positive amnion epithelial and mesenchymal cells from preterm (A) and term (B) placenta 16 hours after the lesion in control and treated cultures (mean percentage ± SD) of two preterm and two term experiments. Proliferating cells were detected in the perilesional area and at the culture edge (A and B).

Comment

This study was performed to examine the repair potential of amnion epithelial and mesenchymal cells from preterm and term placenta and test the effect of potential proliferation stimulants. We found that epithelial and mesenchymal cells differed in repair potential, as did mesenchymal cells from preterm and term placentas, achieving 80 % repair at 40 h versus 40 %, respectively. Thus mesenchymal cells from term placenta healed more slowly than their preterm counterparts.

We found no enhancement of lesion repair after treatment with four potential stimulants of cell proliferation and migration: PDGF, TNFα, fibrinogen and PMA. No factor increased preterm epithelial proliferation versus control. However, both PDGF and TNFα stimulated preterm mesenchymal proliferation. This finding is inconsistent with the incomplete mesenchymal repair observed at 40 h in the stimulated and control groups. A possible explanation is that PDGF and TNFα did stimulate proliferation but a decrease in the size of the lesion could not be detected within our 40 h study period due to very long doubling time of amnion cells in culture (150-210 h[86]). However, PDGF and TNFα did not stimulate the proliferation of term mesenchymal cells, which was also approximately double that of preterm mesenchymal cells (control group).

No data have previously been published on the repair capacity of isolated human amnion epithelial and mesenchymal cells from preterm and term placenta. Earlier studies focused either on organ cultures for evaluating the healing capacity of fetal membrane[28] or on the repair kinetics of the FL human cell line[106]. The latter was established by HeLa cell contamination (American Type Culture Collection:

www.atcc.org), suggesting that the cell population used in the study was not precisely defined.

To our knowledge ours is the first study of lesion repair by isolated amnion epithelial and mesenchymal cells using an *in vitro* lesion repair assay. *In vitro* characterization is an essential prerequisite for understanding the basic mechanisms by which healing occurs. The assay was conducted in primary monolayer cell cultures as these have been shown to share several morphologic, biochemical, and enzymatic characteristics with cells in amnion tissue[81]. Further study is now required using different extracellular matrix molecules. There are at least three mechanisms via which the extracellular matrix can regulate cell behaviour through specific morphogenetic signals: 1) the composition of the matrix itself; 2) synergistic interaction between matrix molecules and growth factors; and 3) cell surface receptors mediating adhesion to matrix components[89]. We also need to evaluate the effects of lesion type and size on amnion cell repair processes at different gestational ages. The present study is simply a first step towards characterizing the repair capacity of amnion epithelial and mesenchymal cells of different gestational age with a long-term view to their use in the development of new therapeutic strategies for PROM.

Acknowledgment

We thank Tilo Burkhardt, MD, from the Zurich University Hospital and Prisca Schaer-Zammaretti, PhD, from the Institute for Biomedical Engineering, ETH and University of Zurich, Switzerland, for their critical review of the manuscript.

Chapter 4

Human preterm amnion cells cultured in three-dimensional collagen I and fibrin matrices for tissue engineering purposes

In Press: Am J Obstet Gynecol, 2005

Abstract

Objective: In this study, human preterm amnion cells were investigated in three-dimensional (3D) cell-matrix culture systems in an attempt to design therapeutic strategies for preterm premature rupture of the membranes.

Study design: 3D collagen I and fibrin cell containing biomatrices were created on a way that mimics the architecture of native amnion. Amnion mesenchymal cells were embedded in 3D matrices and epithelial cells were placed on top of these matrices. Cell viability and morphology were visualized by DiI-ac-LDL, F-actin and nuclear staining. Proteolytic activity of matrix metalloproteinases (MMPs) was investigated using gelatine zymography.

Results: Preterm amnion epithelial and mesenchymal cells cultured in collagen I and fibrin matrices assume cell morphologies, similar to those observed in vivo. Mesenchymal cells were capable of remodelling collagen I, as seen by extensive volume contraction by 40 % at day 1 and 80 % at day 5. Matrix contraction was independent of the presence of epithelial cells, and could not be inhibited by GM6001 and/or aprotinin. No contraction was observed in fibrin matrices over 8 days. The migratory response of mesenchymal cells cultured in 3D fibrin matrices supplemented with fibronectin was associated with specific activated MMP-9.

Conclusion: 3D fibrin matrices might be useful in amnion cell tissue engineering including cell-matrix transplantation.

Key words: amnion epithelial and mesenchymal cells, three-dimensional cell-matrix system, collagen I, fibrin, MMPs

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Introduction

Preterm premature rupture of the membranes (PPROM) occurs in approximately 1 % of all pregnancies and is associated with 30-40 % of preterm deliveries. It is the leading identifiable cause of preterm delivery[5]. Both PPROM and preterm birth carry a high risk of maternal morbidity and neonatal morbidity and mortality[98]. Several treatments for premature rupture of the membranes (PROM) in human fetal membrane models have been proposed. The application of maternal blood clot patches has been recognized as a potential option for therapy of PPROM[29]. However, the intra-amniotic deposition of a mixture of platelets in fibrin cryoprecipitate called 'amniopatch'[30, 99, 100] as well as a trans-vaginally applied intracervical fibrin sealant[40] have been only partially successful in treatment of PPROM. A recent single case report for treatment of PPROM refers to using a collagen plug in combination with additional fixation with fibrin glue adhesives[46]. Although the sealing was initially effective, PPROM reoccurred after two weeks. Despite their apparent potential, none of these treatments have been introduced into routine clinical use. Failure of those trials may have resulted from the lack of integration of the plugs at the site of regeneration and the lack of subsequent healing responses. Based on the literature, the healing responses after implantation of the plug strongly depend on recruitment of cells from adjacent native tissue. In addition, the healing process may be facilitated and accelerated by tissue specific cells provided within a biocompatible plug on/in which these cells can proliferate[48, 107].

The aim of this study was to investigate the behaviour of human amnion cells in three-dimensional (3D) collagen I and fibrin matrices, with the ultimate objective to develop a clinically relevant cell-matrix system for treatment of PPROM.

Material and methods

Isolation and culture of human preterm amnion epithelial and mesenchymal cells

Preterm amnion epithelial and mesenhymal cells were isolated and cultured as described previously[108]. Amnion tissues were collected immediately after elective cesarean section from six preterm (28-36 week) placentas with attached membranes from women without PROM, signs of infection or chromosomal abnormalities. The main indications for the elective cesarean sections were preeclampsia, intrauterine growth retardation and placenta praevia. Amnion and chorion leave tissues were separated by blunt dissection[81, 104], and the amnion was cut approximately 2 cm from the placental disc to avoid the 'zone of altered morphology'[82, 105]. The samples were washed in phosphate buffered saline (PBS; Sigma, Buchs, Switzerland) to remove cellular debris and blood, minced and treated four times with 0.25 % trypsin (Gibco BRL, Basel, Switzerland) for 15 min. The first digestion supernatant, consisting primarily of red blood cells, was discarded. Epithelial cells obtained during subsequent trypsin treatments and were collected by centrifugation at 1000 rpm for 5 min and resuspended in a basal medium comprising a 1:1 mixture of Ham's F-12 and Dulbecco's minimal essential medium (Gibco) containing 10 % heat inactivated fetal bovine serum (FBS; Gibco), 100 U/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco). Mesenchymal cells were isolated from the remaining amnion tissue after complete (>98 %) removal of epithelial cells (evaluated microscopically). The tissue was minced and incubated with 2 mg/ml collagenase A (Roche Diagnostics, Rotkreuz, Switzerland) at 37° C for 120 min. Dispersed mesenchymal cells were collected by centrifugation at 1000 rpm for 5 min. Epithelial and mesenchymal cell viability as assessed by trypan blue dye exclusion and

was found to be over 97 %. Both cell types were cultured further in basal medium supplemented with 50 ng/ml epidermal growth factor (EGF; Sigma), 2.5 μg/ml insulin (Sigma), 5 μg/ml transferrin (Sigma), and 0.1 ng/ml triiodothyronine (T_3; Sigma). This medium is further referred to as 'supplemented basal medium'. The cells were maintained in culture until confluence in a humidified atmosphere at 37° C and 5 % CO_2.

Co-culture of preterm amnion epithelial and mesenchymal cells in three-dimensional collagen I matrices

The 3D collagen I matrices used for co-culture of embedded preterm amnion epithelial and mesenchymal cells were utilized to create a cell-matrix system that mimics the structure of native amnion (Figure 1). 1 x 10^4 preterm amnion mesenchymal cells were mixed with 100 μl neutralized collagen I solution (BD Biosciences, Bedford, MA, USA). The final concentration of collagen I solution was 2 mg/ml. Samples were placed in 96-well MicroWell® plates (Nunclon delta, Nunc, Wiesbaden, Germany). Polymerization was allowed to proceed for 15 minutes at 37º C. Subsequently, 1 x 10^4 preterm amnion epithelial cells were added on top of the collagen I matrices. Cell-matrix systems were cultured in 'supplemented basal medium' at 37º C, 5 % CO_2, in a humidified atmosphere.

Assessment of global collagen I matrix contraction

Eight experimental groups of collagen I matrices were examined as outlined in Table I. These eight experimental groups were performed using epithelial and mesenchymal cells of three different preterm amnion membranes. Mesenchymal cells were either embedded into 3D collagen I matrices (Group I, III, V, VII) or matrices containing mesenchymal cells were covered by epithelial cells (Group II, IV, VI, VIII). In some indicated cases GM6001 (an inhibitor of MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9; Chemicon, Juro Supply GmbH, Lucerne, Switzerland) and/or aprotinin (an inhibitor of serine proteases; Sigma, Switzerland) were mixed into the collagen I solution prior to polymerization at a final concentration of 10 μM and 50 μg/ml, respectively. Native cell-free collagen I matrices were used as controls. The size of 3D collagen I matrices was analyzed by phase contrast and fluorescence microscopy Leica DMIL (Leica, Glattbrugg, Switzerland) equipped with a Leica DC 300F digital camera (Leica, Glattbrugg, Switzerland). Collagen I matrix contraction was monitored daily and processed using a LeicaQ Win Image Analysis software (Leica Imaging System, Ltd., Cambridge, England, UK) and Photoshop®7.0 (Adobe Systems Inc., USA). The size of 3D collagen I matrices were calculated by subtracting the matrix size in square micrometers from the size measured at t=0 (0 hours). Values were expressed as a percentage of the size at t=0 (100 %).

Groups of 3D collagen I matrices	Mesenchymal cells	Epithelial cells	GM6001 (10μM)	Aprotinin (50μg/ml)
I	+	-	-	-
II	+	+	-	-
III	+	-	+	-
IV	+	+	+	-
V	+	-	-	+
VI	+	+	-	+
VII	+	-	+	+
VIII	+	+	+	+
Control	-	-	-	-

Table I. Experimental groups of 3D collagen I matrices. Mesenchymal cells were embedded into collagen I matrices (Group I, III, V, VII) or mesenchymal cells were embedded and epithelial cells were seeded on top of these matrices (Group II, IV, VI, VIII). In some indicated cases the matrices were incubated with 10 μM GM6001 (Group III, IV, VII, VIII) and/or with 50 μg/ml aprotinin (Group V, VI, VII, VIII). Native cell-free collagen I matrices were used as control.

Preparation of fibrin matrices

Fibrin matrices were prepared according to standard protocols by mixing the following components to the final concentrations: 2 mg/ml fibrinogen (Fluka AG, Buchs, Switzerland) in 10 mM Tris bufferred saline, pH 7.4, 0.5 U/ml factor XIII (kindly provided by Baxter AG, Vienna, Austria), 2 NIH units/ml human thrombin (Sigma), and 2.5 mM $CaCl_2$. Collagen I, fibronectin (Bioreba, Basel; Switzerland) or laminin-1 (Sigma) were included at 20 μg/ml gel prior to initiation of fibrin polymerization by addition of thrombin (Sigma).

Co-culture of preterm amnion epithelial and mesenchymal cells in fibrin-based hydrogel matrices

3D fibrin matrices were performed using epithelial and mesenchymal cells from three preterm amnion membranes. 2 x 10^4 preterm mesenchymal cells were suspended in 200 µl fibrinogen solution containing different extracellular matrix molecules at 20 µg/ml prior to initiation of fibrin polymerization. Samples were placed in 8-well glass culture dishes (Nunc Lab-Tek^{TM}II Chamber slide system; VWR International AG, Dietikon, Switzerland). Polymerization induced by thrombin was allowed to continue for 5 min at 37º C. Subsequently, the surface of 3D fibrin matrices were coated with either collagen I, fibronectin or laminin at 20 µg/ml and allowed to polymerize for another 1 h at 37º C. Thereafter, 2 x 10^4 preterm amnion epithelial cells were seeded on top of the 3D matrices (Figure 1). Cells-matrix systems were cultured for 8 days as described above.

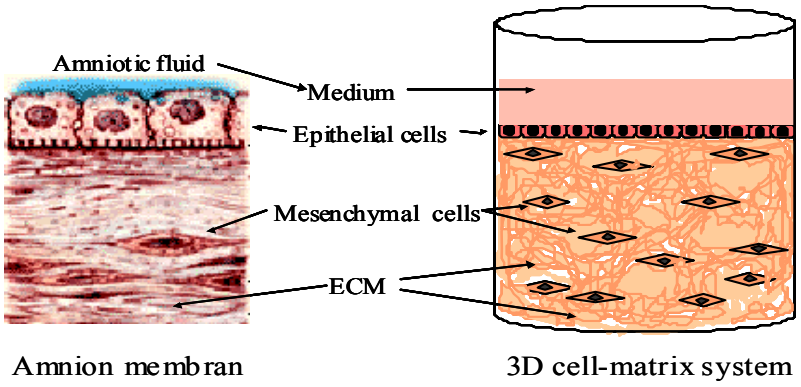


Figure 1. Schematic illustration of the natural term amniotic membrane (left). The schematic representation on the right depicts our cell-culture model that aims to mimic the cellular and structural composition of natural amnion. Preterm amnion mesenchymal cells were embedded in 3D matrices and epithelial cells were placed on top of these 3D matrices.

Cytochemical analysis

To detect the uptake of 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine-labeled acetylated LDL (DiI-ac-LDL; BTI Inc., Stoughton, MA), preterm amnion epithelial and mesenchymal cells were incubated with DiI-ac-LDL (2.4 µg/ml) at 37° C for 1 h. Cells were then fixed with 2 % paraformaldehyde for 10 min, washed 3x with PBS, permeabilized with 0.2 % Triton X-100 and incubated with rhodamine-labelled phalloidin (Molecular Probes, Eugene, OR) in order to visualize F-actin filaments. Finally, 4'6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Eugene, OR) was used to stain the nuclei of all cells. The fluorescently labeled cells were imaged with confocal laser scanning microscopy (Leica SP2, Leica, Glattbrugg, Switzerland). In some indicated cases, the data were further processed using Imaris software (Bitplane AG, Zurich, Switzerland).

Gelatin zymography

Gelatin zymography was used to investigate the proteolytic activities of MMPs expressed by preterm amnion mesenchymal cells embedded into 3D fibrin matrices. Collagen I, fibronectin or laminin-1 was included into fibrin matrices prior to coagulation. Native fibrin matrices without additional extracellular matrix molecules were used as controls. The culture supernatants were collected at day 1, 3, 5, and 7. The gelatinolytic activities of MMPs in cell-matrix culture supernatants were assessed as previously described[109]. Briefly, 10 µg of total proteins were applied onto 10 % SDS-polyacrylamide gels copolymerized with 1 mg/ml gelatin and electrophoretically separated. Recombinant human MMP-2 and MMP-9 (Chemicon International, Inc., Temecula, CA) were used as gelatine zymography standards. The gels were

subsequently washed twice for 20 min with 2.5 % Triton X-100 to remove SDS and to allow the MMPs to renature before incubating in zymography buffer (20 mM Tris, pH 8.0, 5 mM calcium chloride, 0.02 % sodium azide). The gels were incubated for 20 h at 37° C with agitation. The zymographic gels were scanned using a Silverfast Expr1460 scanner equipped with SilverFast v5.52r09 software (LaserSoft Imaging AG, Germany) and compared with each other.

Statistical analysis

Mean values and standard deviation are reported. Differences between two groups were analysed using the Mann-Whitney U test. The Kruskal-Wallis test was used to test the differences between several groups. Significance level was set on $p<0.05$.

Results

Preterm amnion mesenchymal cells contract collagen I matrices

The use of amnion cell cultures in collagen I matrices was challenged by the extensive collagen I matrix contraction mediated by amnion mesenchymal cells. A significant decrease in the size of anchored collagen I matrices was detected over the course of 6 days ($p<0.001$). In fact, the size of collagen I matrices was reduced by 40 % at culture day 1 and 80 % at culture day 5. This effect was independent of the presence of co-cultured preterm amnion epithelial cells (Figure 2A). Furthermore, collagen I contraction could not be blocked by GM6001(10 μM) or/and by aprotinin (50 μg/ml) (Table II). In the absence of amnion cells, no change in collagen I matrix size was detectable (Figure 2A). Additionally, migration of living preterm amnion mesenchymal

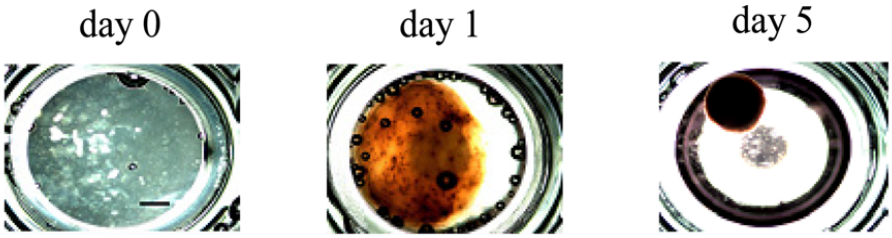
cells out of zones of dense collagen I into zones of low collagen I concentrations was observed (Figure 2B).

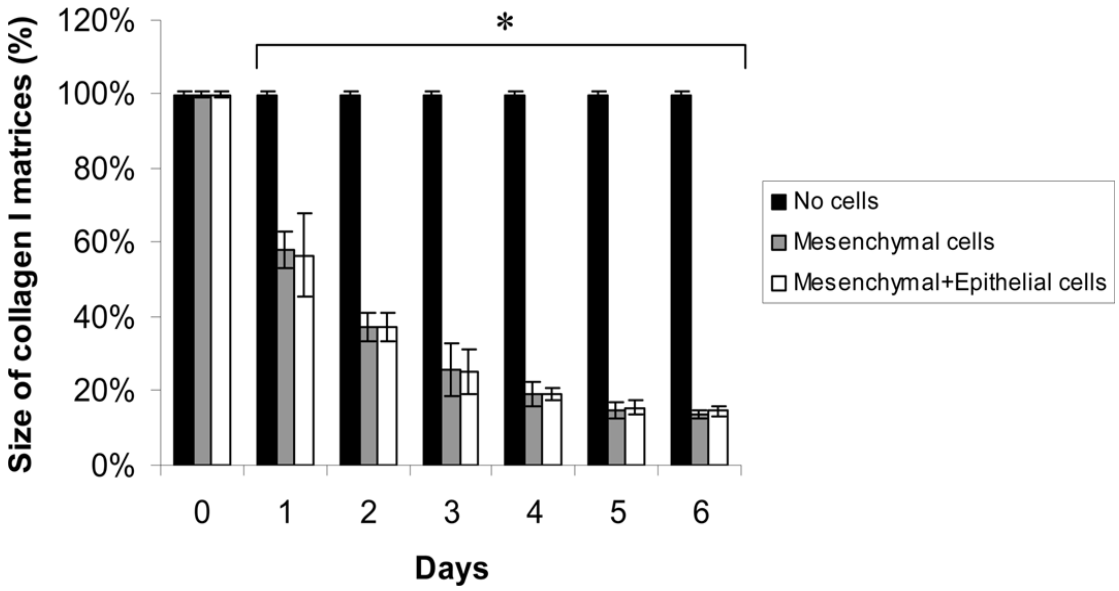
In summary, collagen I matrices significantly contract with increasing culture time, and preterm amnion mesenchymal cells are capable to remodel 3D collagen I matrices and to migrate out of 3D collagen I matrices.

	Average of collagen I matrix size (%)			
Groups of 3D collagen I matrices	Day 0	Day 1	Day 6	Matrix contraction
I	99±1	58±5	14±1	yes
II	99±1	57±11	15±1	yes
III	99±1	66±5	25±7	yes
IV	99±1	65±7	26±9	yes
V	99±1	60±6	29±6	yes
VI	99±1	61±8	28±6	yes
VII	99±1	63±8	27±7	yes
VIII	99±1	64±6	26±6	yes
Control *	99±1	99±1	99±1	no

Table II. Contraction of collagen I matrices mediated by preterm amnion mesenchymal cells (Group I and II) could not be blocked by 10 μM GM6001 and/or 50 μg/ml aprotinin (Group III, IV, V, VI, VII and VIII). In the absence of amnion cells (Control), no change in collagen I matrix size was observed. * indicates values that are significantly different from cell containing collagen I matrices. Matrix sizes are expressed as percentages of initial matrix size at day 0, and are given as mean ± SD of three experiments.

A


day 0
day 1
day 5


Size of collagen I matrices (%)
120%
100%
80%
60%
40%
20%
0%
*
0
1
2
3
4
5
6
Days
No cells
Mesenchymal cells
Mesenchymal+Epithelial cells

B

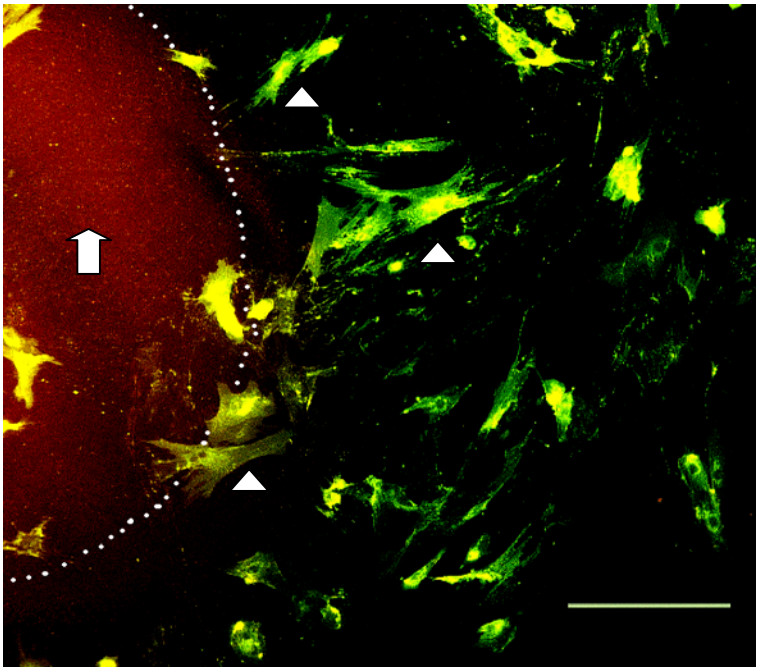


Figure 2. (A) Contraction of collagen I matrices mediated by preterm amnion mesenchymal cells. The size of collagen I matrices was reduced by 40 % at day 1 and 80 % at day 5. This effect was independent of the presence of co-cultured preterm amnion epithelial cells. In the absence of amnion cells, no change in collagen I matrix size was observed. Matrix sizes are expressed as percentages of initial matrix size at day 0, and are given as mean ± SD of three experiments. * indicates statistical significance of the condition 'cell free-collagen I matrices' vs condition 'collagen I matrices containing mesenchymal and epithelial cells' ($p<0.001$). Scale bar: 1mm. (B) Migration of living preterm amnion mesenchymal cells (arrowhead) from a zone of dense collagen I matrix (arrow) into zones lacking collagen I after contractions of the matrix. The dashed line indicates, approximately, the borders of contracted collagen I matrix. Living migrating cells were stained with DiI-ac-LDL. Scale bar: 250 µm.

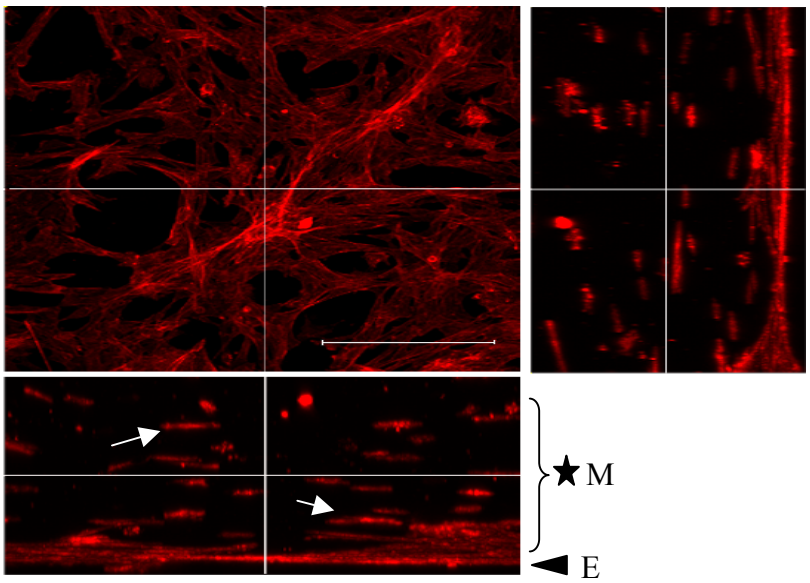
Co-culture of preterm amnion epithelial and mesenchymal cells within native and modified fibrin hydrogel matrices

As illustrated in Figures 3A-C fibrin matrix supplemented with fibronectin maintained the overall morphology of co-cultured amnion epithelial and mesenchymal cells. The overall cell morphology was visualised by co-staining for both F-actin and for cell nuclei. At culture day 2, amnion mesenchymal cells were sparsely distributed within the matrix and lacked prominent extensions (Figure 3A). In contrast, at culture day 8 amnion mesenchymal cells displayed formation of the typically migratory phenotype with long cellular extensions (Figures 3B-C). As judged by DAPI staining, the number of mesenchymal cells was not significantly changed with increasing culture time ($p=0.556$). At culture days 2 and 8, the mean number of mesenchymal cells was 135 ± 7 cells/0.5 mm^2 and 140 ± 14 cells/0.5 mm^2, respectively (Figure 3D). This finding indicates that preterm amnion mesenchymal cells in 3D fibrin matrices undergo differentiation rather than proliferation. Furthermore, fibrin matrices supplemented with fibronectin maintained the vitality of amnion cells. The vitality of amnion cells was not significantly changed over the time course of 8 days ($p=0.444$). At culture day 2, 87 % ± 9 % of embedded mesenchymal cells (Figure 3D) and 89 % ± 7 % of epithelial cells were positive for DiI-ac- LDL uptake, determined as an indicator for cell-viability. Consistently at day 8, 84 ± 11 % of mesenchymal cells (Figure 3D) and 86 % ± 5 % of epithelial cells displayed viability. The amnion epithelial cell layer was formed on top of 3D fibrin matrices supplemented with fibronection. Amnion epithelial cells appeared similar to those observed *in vivo* (data not shown). Notably, no significantly decreased in the size of fibrin matrices was detected over the course of 8 days ($p=0.222$) (Figure

3E). Additionally, the overall morphology of amnion epithelial and mesenchymal cells cultured in fibrin matrices supplemented either with collagen I or laminin-1 was similar as in fibrin matrices supplemented with fibronectin (data not shown).

Taken together, preterm amnion epithelial and mesenchymal cell morphologies in 3D fibrin matrices were found to be similar as observed *in vivo*. Amnion cells are highly viable and no significantly decrease in the size of fibrin cell-matrix systems was observed.

A



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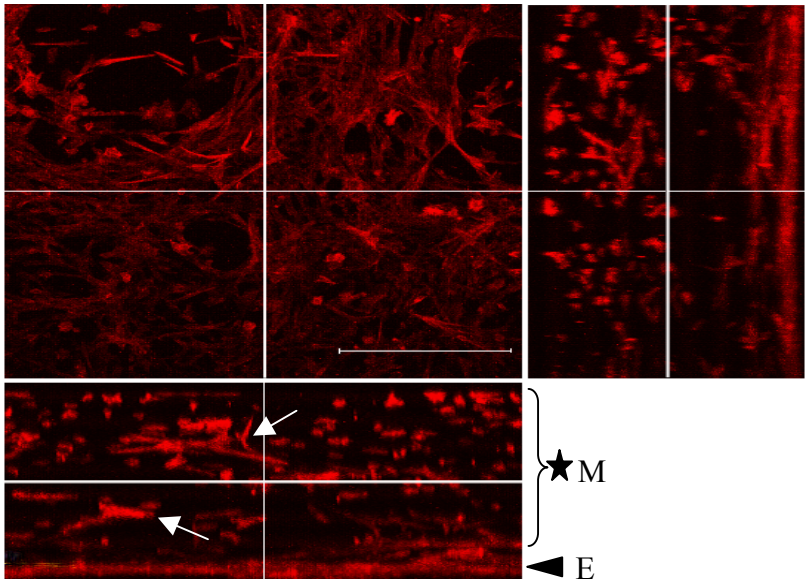

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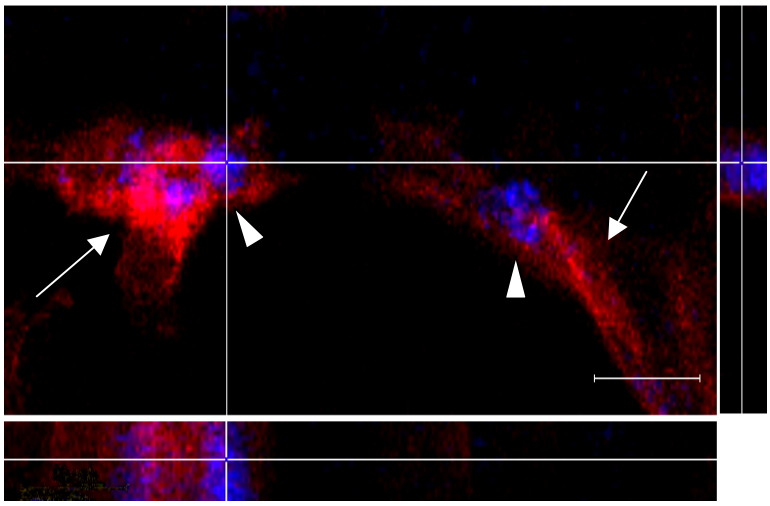

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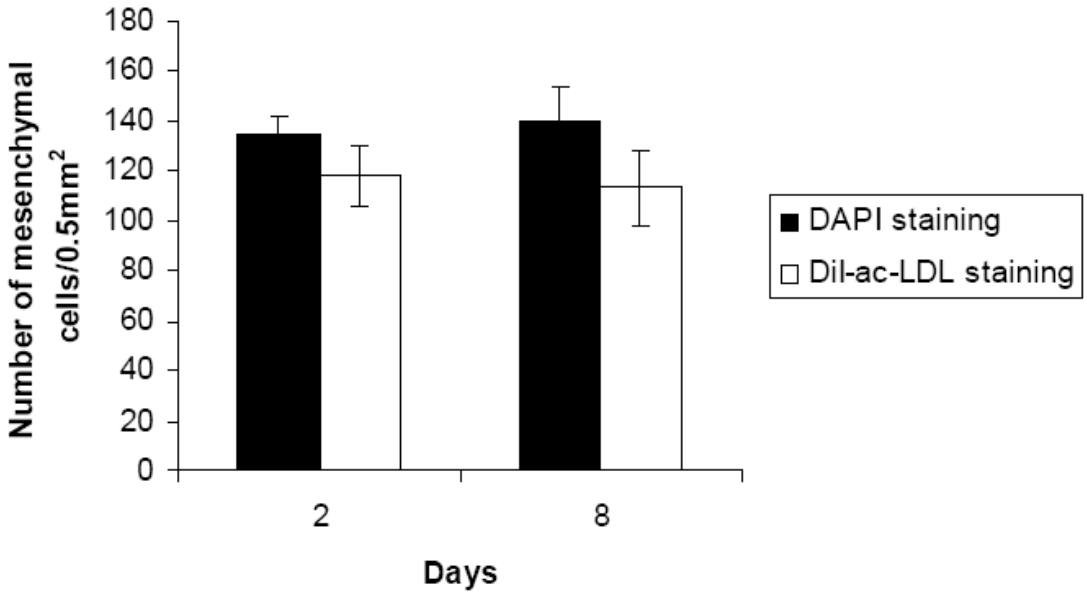
Number of mesenchymal cells/0.5mm²
180
160
140
120
100
80
60
40
20
0
2
8
Days
DAPI staining
Dil-ac-LDL staining

E

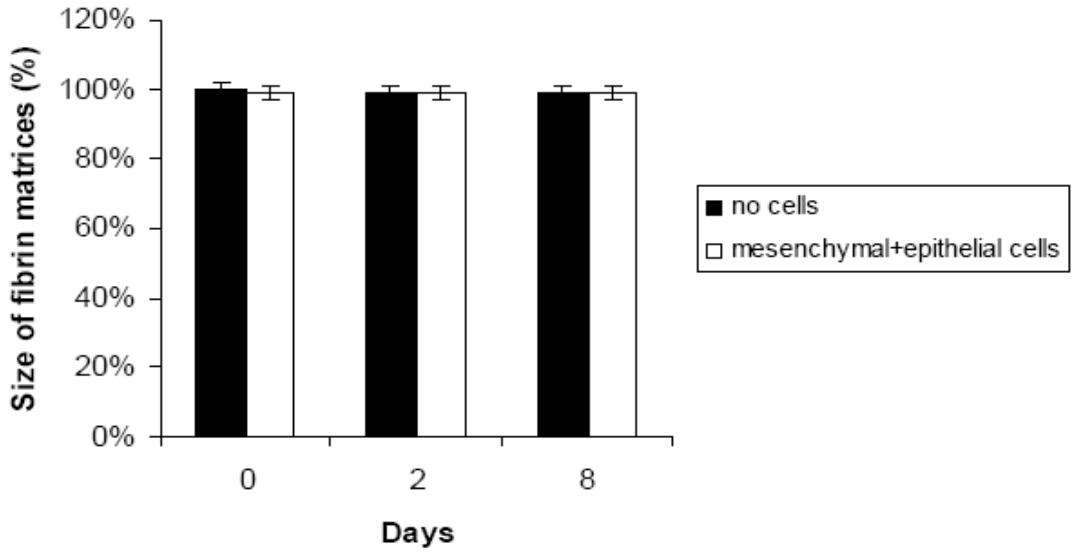

Figure 3. Morphology of preterm amnion epithelial and mesenchymal cells in 3D fibrin matrices supplemented with fibronectin. Overall cell morphology at culture days 2 and 8 was visualized by staining for F-actin in combination with a DAPI nuclear stain. The reconstruction of the 3D system was performed using a stacked series of optical slices obtained by confocal laser scanning microscopy. (A) Amnion cell morphology visualized at culture day 2. Amnion mesenchymal cells were sparsely distributed without prominent extensions (M; arrow; star). The position of amnion epithelial cells is indicated (E; arrowhead). (B) Amnion cell morphology visualized at culture day 8. Amnion mesenchymal cells displayed a formation of typically migratory phenotype with cell extension (M; arrow; star). The position of amnion epithelial cells is indicated (E; arrowhead). (C) Morphology of preterm amnion mesenchymal cells at higher

magnification at culture day 8. Overall mesenchymal cell morphology was visualised by combining staining for F-actin (arrow) and DAPI staining for nuclei (arrowhead). (D) Amnion mesenchymal cells were highly viable as determined by Dil-ac-LDL uptake and the cell number determined by DAPI stain was not significantly changed over the time course of 8 days ($p=0.556$). Data represent evaluation of three images per culture condition. (E) No significantly decreased in the size of fibrin matrices was detected over the 8 days ($p=0.222$). Scale bars: A, 500μm; B, 500μm; C, 40μm.

Preterm amnion mesenchymal cell migration within fibrin matrices supplemented with fibronectin is associated with activation of MMP-9

The migratory response of preterm amnion mesenchymal cells cultured in fibrin matrices supplied with fibronectin was associated with increased levels of activated MMP-9. This activity was reduced after incubation with 10 μM GM6001. However, no activated MMP-9 was detected in cultures of preterm amnion mesenchymal cells within native fibrin matrices, or fibrin matrices containing collagen I or laminin-1. The inactive pro-forms of MMP-9 and MMP-2 could be detected in all cultures (Figure 4).

In summary, fibronectin filled fibrin matrices were associated with upregulated MMP-9 activation. This seems to be specific for fibronectin as fibrin matrices supplemented with laminin-1 or collagen I did not upregulate MMP-9 activation.

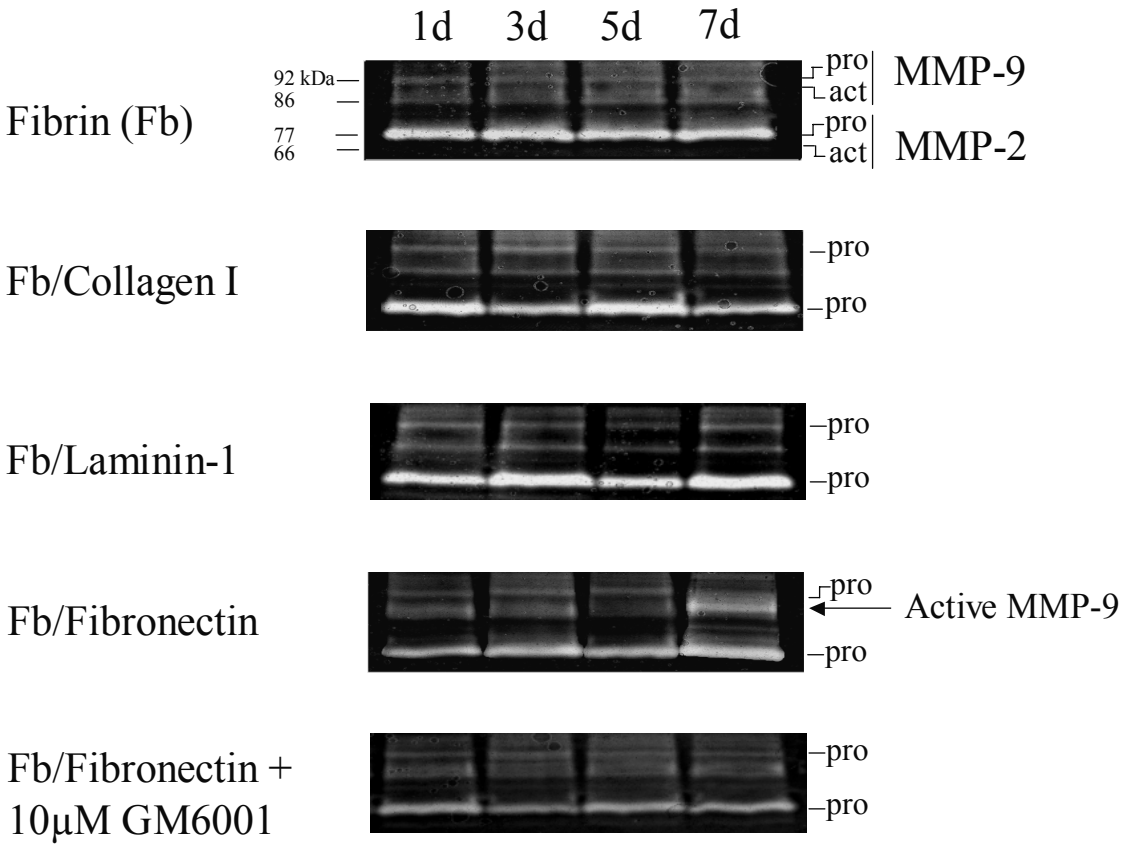

Figure 4. Comparison of gelatinoloytic activity of MMP-9 in cultures of preterm amnion mesenchymal cells cultured within 3D fibrin matrices. Fibrin matrices supplemented with fibronectin, collagen I or laminin-1 were assessed. Control cultures were prepared in native fibrin matrices. Increased levels of activated MMP-9 were detected in fibrin matrices containing fibronectin. Activity of MMP-9 was reduced after treatment with 10 μM GM6001. The positions of pro- and active forms of MMP-2 and MMP-9 are indicated as assessed by comparison with a MMP-2 and MMP-9 marker.

Comment

Our study was performed to investigate the behavior of human preterm amnion epithelial and mesenchymal cells in 3D collagen I and fibrin matrices with the ultimate objective to develop a potential option for the treatment of PPROM patients based on tissue engineering. Tissue engineered transplants can be composed of two components, tissue-specific cells placed into a biocompatible scaffold on/in which these cells can proliferate. The scaffold material provides initial mechanical support and a template for three-dimensional organization[47]. The approach pursued here, relies on the design of a cell containing matrix that mimics the architecture of native amnion. The selection of cellular components of 3D matrices needs to be tissue specific. In our previous study, we have demonstrated that amnion epithelial and mesenchymal cells differ in their repair potential. Mesenchymal cells from preterm placenta healed more quickly than their term counterparts[108]. Consequently, in this study, the cellular components of the here developed cell-matrix transplants were amnion epithelial and mesenchymal cells from preterm placenta. Besides the selection of the cellular components that ultimately determined the function of such cell-matrix systems, an important feature of these systems, is the possibility of *in situ* application. Moreover, concerning the aim of this study, it is important that, such cell-matrix systems can be precisely administered via endoscopical methods to the ruptured site of the amnion membrane. Trying to meet this requirement, we have used 3D fibrin matrices as a scaffold matrix for isolated preterm amnion epithelial and mesenchymal cells. As outlined in this study, two interesting features of the 3D fibrin-matrix system were the refinement by the addition of extracellular matrix molecules that introduce additional adhesion sequences involved in

cell adhesion and/or migration, and specific matrix degradation by cell-associated proteases such as MMPs or serine proteases such as plasmin. We have demonstrated that preterm amnion epithelial and mesenchymal cells in modified 3D fibrin based cell-matrix systems acquire their natural morphologies and displayed viability. In addition, in fibrin matrices supplemented with fibronectin, preterm amnion mesenchymal cells activated proteolytic programs of tissue repair, as specifically demonstrated by the activation of MMP-9. Moreover, fibronectin, here supplemented into native fibrin matrices, is an intrinsic component of clinical 'fibrin glue' formation. During clotting, plasma fibronectin incorporates to the fibrin network. Furthermore, the use of collagen I as a scaffold matrix was also investigated in this work. One limitation of this approach, as observed in this study, is contraction and consequently size reduction of collagen I matrices in vitro. *In vivo*, such a size reduction would inevitably induce failure of the implant followed by clinical consequences. According to the literature, control of the contraction and organization of cells and matrix, can be critical for successfully creating tissue engineered grafts[110]. Unfortunately, these aspect cannot be easily predicated at the design of cell-containing collagen I matrices. In contrast to the collagen I matrix-cell systems, no decrease in the size of fibrin cell-matrix systems was observed over the time course of one week. Related to this data, one might speculate that such fibrin based cell-matrix systems may be useful in amnion cell transplantation. By virtue of fibrin's biological characteristics, such an implant could seal the membrane leak instantly. Subsequently, the fibrin clot may be remodelled into native membrane tissue by amnion cells provided in the matrix as well as by surrounding amnion cells. These fibrin based cell-matrix systems should induce only a low immunological response. First, the fibrin

matrix is physiologically and therapeutically relevant. It is widely applied in surgery as sealant and adhesive, and when formed from pure fibrinogen, represents a highly defined substrate. Secondly, human amnion epithelial cells do not express any of the leukocyte antigens (HLA)-A, -B, -C or –DR[7], suggesting amnion tissue is a promising cell source for a variety of tissue engineering applications including amnion cell transplantation. Recently, successful xenotransplatations have been reported with human amnion epithelial cells in the spinal cord of monkeys[111]. Furthermore, different clinical studies confirmed that amnion material effectively facilitates epithelialization, can maintain normal cellular phenotypes, and reduce inflammation and scarring. Many studies are ongoing to investigate and use this unique feature of amniotic membranes as a prerequisite for tissue engineering[112]. Although the data presented here is promising, further studies must be undertaken to investigate the response of amnion cell-based tissue engineered grafts to more complex multifactorial *in vivo* environment.

Acknowledgment

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Chapter 5

General discussion and outlook

General discussion and outlook

In the present thesis, a strategy to develop a potential option for the treatment of PPROM patients based on a tissue engineering approach was proposed. Tissue engineered transplants can be composed of two components, tissue-specific cells placed into a biocompatible scaffold on/in which these cells can proliferate. The scaffold material provides initial mechanical support and a template for three-dimensional organization. The approach pursued here, relies on the design of a cell containing matrix that mimics the architecture of native amnion. The selection of cellular components of 3D matrices needs particular attention on a issue. Further issues include establishment of reproducible methodology that permits to collect and expand human amnion epithelial and mesenchymal cells from preterm and term placenta (Chapter 2) as well as investigation of their repair potential *in vitro* (Chapter 3). In brief, it was demonstrated that the repair potential of human amnion epithelial and mesenchymal cells as well as their reaction towards different proliferating stimulants depends on the gestational age. Therefore, this thesis provides experimental data that supports the use of preterm amnion cells for the creation of cell-matrix transplants. This correlates with findings showing that, other characteristics related to the functional and anatomical integrity of the fetal membranes, like tensile strength and ECM composition have meanwhile also shown to be gestational age dependent[113]. Notably, according to the experience made in the course of this thesis, the cultivation of amnion cells can be difficult and may result in insufficient cell viability and cell number. One of the basic step in preparing human amnion cell cultures is collection of primary amnion tissue. It is very important to exclude membranes with any signs of PROM, infections, fetal chromosomal

abnormalities, intrauterine growth retardation , and intrauterine fetal death. This largely affected the viability, survival and proliferation rates of amnion cells. Besides these limitations, the gestational ages can also negatively affect the proliferation potential, especially of term amnion epithelial cells (Chapter 2).

Another basic question that occurred during this work, concerns the selection of the scaffold matrix. Besides the selection of the cellular components that ultimately determined the function of such cell-matrix systems, an important feature of these systems, is the possibility of *in situ* application. Moreover, concerning the aim of this thesis, it is important that, such cell-matrix systems can be precisely administered via endoscopical methods to the ruptured site of the amnion membrane. Trying to meet this requirements, 3D fibrin matrices were used as a scaffold matrix for isolated preterm amnion epithelial and mesenchymal cells. As outlined in this thesis, an interesting refinement of the 3D fibrin-matrix system could be the addition of peptide sequences involved in cell adhesion and/or migration, and being degradable by cell-associated proteases such as MMPs or serine proteases such as plasmin. It was demonstrated, that preterm amnion epithelial and mesenchymal cells in modified 3D fibrin based cell-matrix systems acquire their natural morphologies and displayed viability (Chapter 4). Beside the modification of fibrin matrices by admixtures of cell adhesions factors, as shown in this thesis, their further design improvements could be performed by exogenous admixture of growth factors. These bioactive molecules are produced by the cells themselves, but can as well be added exogenously to such cell-matrix systems to enhance their integration at the site of regeneration and also to induce stronger and more specific healing responses. Some promising factors could be PDGF and TNFα, that

have been found to increase the proliferation of preterm amnion mesenchymal cells (Chapter 3). Furthermore, the use of collagen I as a scaffold matrix was also investigated in this work. Limitation of this approach, as observed in Chapter 4, include contraction and consequently size reduction of collagen I matrices *in vitro*. *In vivo* such size reduction would inevitably induce failure of the implant followed by clinical consequences. According to the literature, control of the contraction and organization of cells and matrix, can be critical for successfully creating tissue engineered grafts[110]. Unfortunately, these aspect cannot be easily predicated at the design of cell-containing collagen I matrices. In contrast to the collagen I matrix-cell systems, no decrease in size of fibrin cell-matrix systems was observed over the time course of one week. Related to this data, one might speculate, that such fibrin based cell-matrix systems may be useful in amnion cell transplantation. By virtue of fibrin's biological characteristics, such an implant could seal the membrane leak instantly. Subsequently, the fibrin clot may be remodeled into native membrane tissue by amnion cells provided in the matrix as well as by surrounding amnion cells. These fibrin based cell-matrix systems may represent a novelty in therapy of PPROM. However, these data are clearly preliminary and have to be confirmed by exposure of amnion cell-based tissue engineered grafts to more complex multifactorial *in vivo* conditions. Such an *in situ* delivery has not been investigated yet. One of the major problems in the design of an in vivo study includes the selection of appropriated animal models. Based on the literature only non-human primates closely resemble the human situation in terms of size, placentation and reproductive biology. Such future animal experiments should be undertaken in order to investigate the impact of a spontaneous cell migration and population on the formation

of tissue engineered constructs. This course of action strongly depends on the ability of cells to migrate across this provisional matrix as well as on their capacity to actively remodel their environment[48, 114]. An additional aspect to be considered for *in vivo* application of these cell-matrix systems is the potential induction of immunological responses. According to the findings in the literature, these fibrin based cell-matrix systems induce only a low immunological response. First, the fibrin matrix is physiologically and therapeutically relevant. It is widely applied in surgery as sealant and adhesive, and when formed from pure fibrinogen, represents a highly defined substrate. Secondly, human amnion epithelial cells do not express any of the leukocyte antigens (HLA)-A, -B, -C or -DR[7] suggesting amnion tissue as a particular promising cell source for a variety of tissue engineering applications including amnion cell transplantation. Recently, successful xenotransplatations have been reported with human amnion epithelial cells in the spinal cord of monkeys[111]. Furthermore, different clinical studies confirmed that amnion material effectively facilitates epithelization, can maintain normal cellular phenotypes, reduce inflammation and scarring. Many studies are undergoing to investigate and use this unique feature of amniotic membranes as a prerequisite for tissue engineering[112]. Besides the promising observations, this issue needs to be well investigated before use in clinical applications. The results presented in this thesis may be only a beginning to gain insight into this poorly studied issue of fetal membrane repair. Although, the interest in fetal membrane repair was stimulated by the increased application of invasive fetal procedures, especially fetoscopy and fetal surgery, resulting in significant incidence in iatrogenic PPROM. Fetal membranes still remain the Achilles' heel of these procedures. A better understanding of biology of fetal

membrane repair on a cellular, enzymatic as well as genetic level, may offer the prospect of developing tissue engineering and/or pharmacological means to modulate fetal membrane repair in order to limit the clinical consequences of spontaneous or iatrogenic PPROM.

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